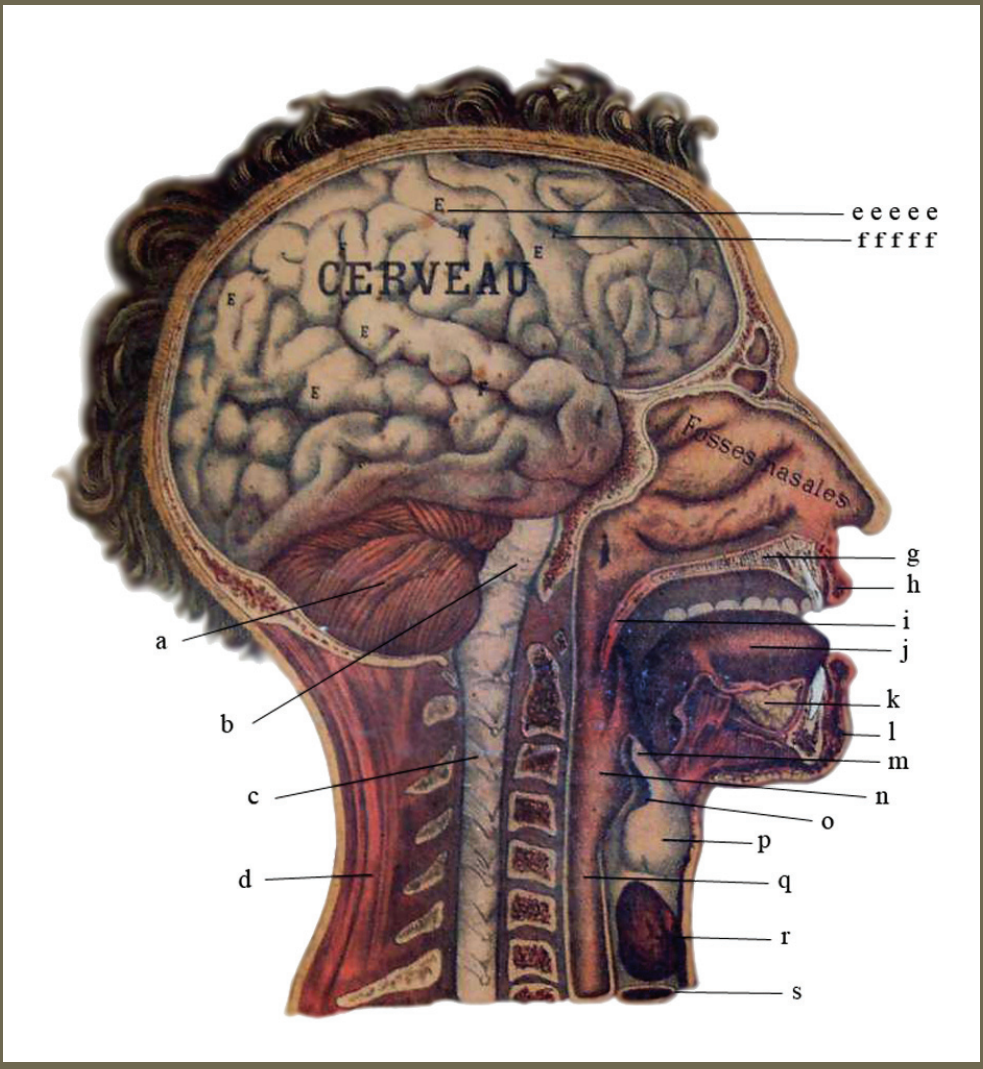
Neuromodulation in Experimental Animal Models of Epilepsy



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Nothing in life is to be feared
It is only to be understood
Marie Curie

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Chapter 1: General introduction

General introduction

Epilepsy is a neurological disorder consisting of recurrent seizures, resulting from excessive, uncontrolled electrical activity in the brain. Despite the pharmacological development of new treatments, still one third of the epilepsy patients does not respond sufficiently to anti-epileptic drugs (AEDs). Those patients are called refractory patients. Hence, there is a constant impetus to search for other treatment strategies like epilepsy surgery, gamma knife surgery, vagus nerve stimulation and deep brain stimulation. Besides the ongoing research on the efficacy of anti-epileptic treatments in suppressing seizures (anti-seizure effect), we want to seek therapies that can lead to plastic changes in the epileptic network and in this way have a modulating effect. The impact of such therapies cannot be overlooked, because they may slow down processes underlying epilepsy, might prevent or even cure epilepsy.

This thesis deals with the potential of neuromodulatory treatments for patients with refractory epilepsy. The treatments that are considered include **the pharmacological therapy with levetiracetam** and **vagus nerve stimulation**. The effect of these therapies was investigated in **chronic models of epilepsy**, which imitate human epilepsy.

1.1 A short introduction to epilepsy

The word ‘epilepsy’ derives from the Greek verb έπιλαμβανειν (epilambanein), meaning ‘to be seized, to be overwhelmed by surprise’. Epilepsy is the most common serious brain disorder affecting 0.5-1% of the general population (Hauser, 1998), but still there is a lot of prejudice and misunderstanding about the disease. Epileptic seizures are characterized by a paroxysmal manifestation of highly synchronized abnormal neuronal activity of a part of the brain (partial) or the whole brain (generalized) resulting in various clinical symptoms which can manifest as motor, sensory, emotional or mixed phenomena possibly with alteration or loss of consciousness. Although 9% of the population experiences a seizure once in a lifetime, epilepsy is only diagnosed when seizures are recurring. The conversion from a normal neuronal network into a hyperexcitable epileptic network is called epileptogenesis, which consists of complex and dynamic processes.

Several epilepsy syndromes exist, but they are all characterized by repetitive seizures. There are multiple causes for epilepsy and seizures can be classified according to their etiology into three categories: *idiopathic* (primary, without a known or suggested genetic origin), *symptomatic* (secondary, resulting from known origins e.g. tumors, lesions, infections, vascular causes) or *cryptogenic* (presumably symptomatic but currently of unknown specific etiology) (Engel and Pedley, 1999). Furthermore, the International League

Against Epilepsy has classified epilepsies according to their seizures onset zone: focal (partial) or generalized.

1.2 Treatment of epilepsy

Epilepsy treatment is indicated following two or more unprovoked epileptic seizures and is successful in the majority of the cases. Despite the pharmacological development of new treatments still, one third of the epilepsy patients do not respond sufficiently to AEDs and are called 'refractory patients' (Kwan and Brodie, 2000). This leads to a constant search for other treatment strategies such as epilepsy surgery, gamma knife surgery, ketogenic diet, deep brain stimulation, vagus nerve stimulation and transcranial magnetic stimulation but also the development of new AEDs with novel mechanisms of action.

Pharmacological treatment is still the most important way to treat epilepsy and newly developed AEDs (e.g. levetiracetam) can provide seizure freedom in up to 7% of refractory patients (Fisher, 1993). AEDs are mainly administered as a long-term treatment to prevent the occurrence of seizures. During the last two or three decades, considerable advances have been made in our understanding of the mechanism of action (MOA) of AEDs (Table 1). This allowed a more rational approach to drug treatment. There are different ways by which AEDs can suppress epileptic seizures: i) decrease in neuronal excitation (glutamatergic system), ii) increase in neuronal inhibition (GABAergic system) or iii) by modulation of ion channels (modification of cellular excitability) (Bradford, 1995). The difficulty of treating epilepsy is that it can involve multifactorial causes and that the use of one approach is often insufficient.

Table 1: Different AEDs with their mechanisms of action (Brodie and French, 2003).

Drug	Na^+ channels	Ca^+ channels	K^+ channels	Inhibitory transmission	Excitatory transmission
Phenytoin	+ + +	+			
Carbamazepine	+ + +				
Sodium valproate	+	+		+ +	+
Ethosuximide		+ + +			
Phenobarbital		+		+ + +	+
Benzodiazepines				+ + +	
Gabapentin	+	+		+ +	
Lamotrigine	+ + +	+			
Oxcarbazepine	+ + +	+	+		
Topiramate	+ +	+ +		+ +	+ +
Tiagabine				+ + +	
Zonisamide	+ +	+ +			
Levetiracetam		+	+	+	+
Felbamate	+ +	+		+ +	+ +
Vigabatrin				+ + +	

Key: + + + = primary action; + + = probable action; + = possible action.

The original goal of pharmacological therapy for patients with epilepsy is to suppress seizures without side effects. In seizure free patients, seizures will mostly remain suppressed as long as the treatment continues, but may reoccur once therapy stops. Anti-epileptogenic

treatment is therefore an attractive idea. However, for the rational development of anti-epileptogenic therapies there is need for a better understanding of epileptogenesis.

Levetiracetam (LEV) is believed to belong to a novel class of AEDs having anti-epileptogenic properties and it was discovered by unconventional drug screening. LEV has a favorable pharmacokinetic profile with rapid absorption following oral administration, excellent bioavailability, quick achievement of steady-state concentrations, linear kinetics and a minimal plasma binding (Patsalos, 2000). The MOA of LEV differs from other AEDs and is as yet not fully elucidated.

Epilepsy surgery is an invasive but often curative treatment option that aims at removing the ictal onset zone believed to be responsible for seizure occurrence (Figure 1).

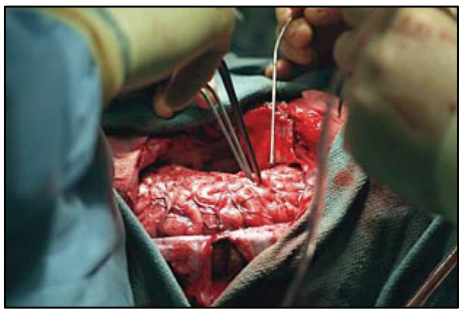

Figure 1: resection of epileptic tissue.

Depending on the underlying epilepsy syndrome, approximately 70% of the patients are rendered seizure free after resective epilepsy surgery (Engel, 1996). The risk-benefit-analysis for surgery must be individualized using a presurgical evaluation protocol and eventually a substantial number of patients have to be rejected (Boon et al., 1999).

A promising alternative is *gamma knife surgery (GKS)*, which is based on the convergence of 201 gamma ray beams. Cortical structures can be targeted with GKS with a stereotaxic precision, without opening the skull and with a limited risk. A recent multicenter study demonstrates that GKS has positive outcomes in patients with epilepsy (Regis et al., 2004).

The *ketogenic diet* is a high-fat, adequate protein, low carbohydrate diet that mimics the biochemical changes associated with starvation, which create ketosis. It appears to be a very effective treatment for epilepsy, particularly during childhood, but has many side effects. It must be prescribed thoughtfully, implemented carefully and monitored closely (Nordli, 2002).

Different sites in the nervous system have been stimulated electrically in an attempt to treat seizures resulting in several types of neurostimulation (Figure 2). *Deep brain stimulation (DBS)* consists of continuous stimulation of specific deep brain regions at the seizure onset zone e.g. amygdala, hippocampus, thalamus (Vonck et al., 2002). On the other hand, electrical stimulation of relay nuclei may have the capacity to influence electrical activity in widespread regions of the brain, useful for multifocal seizure types. DBS is still far from being an established therapeutic technique. Basic research to date has been insufficient to answer questions needed prior to large-scale use of this technique.

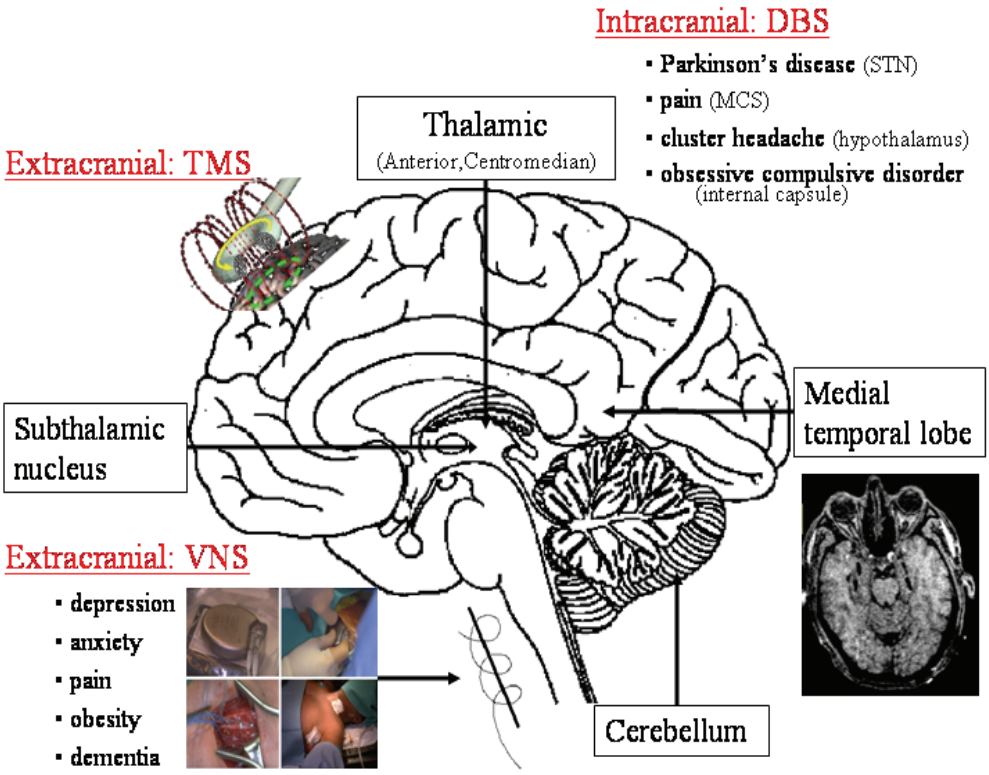

Figure 2: Overview of the different targets for brain stimulation adapted from Vonck (2003). Deep brain stimulation (DBS) is a form of intracranial stimulation, whereas vagus nerve stimulation (VNS) and transcranial magnetic stimulation (TMS) are both forms of extracranial stimulation. Abbreviations: STN, subthalamic nucleus; MCS, motor cortex stimulation.

Vagus nerve stimulation (VNS) is a less invasive extracranial form of stimulation. The left vagus nerve is stimulated intermittently by means of a pulse generator to reduce frequency and severity of epileptic seizures. Controlled randomized studies showed a 50% decrease in overall seizure frequency in approximately one third of the patients, between 30-50% decrease in seizure frequency in another third op patients and, finally, one third of the patients has less than 30% decrease in seizure frequency and are consider to be non-responders (Salinsky, 2003). Through diffuse projections of the vagus nerve in the cerebral hemispheres, VNS can have a broad effect on neuronal excitability (Rutecki, 1990). However, the precise MOA of VNS remains unknown. Several studies in humans describe a cumulative effect of VNS resulting in an increased efficacy over a longer period of time (Vonck et al., 1999; Boon et al., 2001).

Transcranial magnetic stimulation (TMS) with either a hand-held magnet or a frame is used widely in clinical neurophysiology to measure motor-cortex excitability. Recently, low frequency repetitive TMS (RTMS) has been used to reduce motor-cortex excitability (Ziemann et al., 1998). However, clinical experience is limited and only a few epilepsy patients have been treated with RTMS.

1.3 Animal models of epilepsy

The development and characterization of existing and future animal models will lead to greater insight into the pathophysiology underlying epilepsy and will contribute to the evaluation and the development of new anti-epileptic and anti-epileptogenic therapies.

A number of characteristics can be suggested for an ideal animal model imitating human epilepsy such as similar pathology and spontaneous seizures after a period of epileptogenesis. Therefore, chronic models (animals made epileptic or with inborn epilepsy) are preferable to acute models (seizure induction in naïve, healthy (non-epileptic) animals). At present, there are two distinct groups of chronic models: models for *idiopathic* (genetic) epilepsy and models for *symptomatic* (acquired) epilepsy.

A discussion of all possible animal models of epilepsy is beyond the scope of the present thesis. Subsequently, we will be focusing on two chronic models of absence epilepsy (WAG/Rij and genetic absence epilepsy rats from Strasbourg- GAERS) and two chronic models of temporal lobe epilepsy (status epilepticus model and kindling model).

1.3.1 Models of absence epilepsy (idiopathic epilepsy)

It has become increasingly obvious during recent decades that genetic factors play a main role in the idiopathic generalized epilepsies, including absence epilepsy. Absence seizures are characterized by paroxysmal unresponsiveness to environmental stimuli and cessation of ongoing activity. In humans, absence seizures are associated with the appearance of bilaterally synchronous 3 Hz spike and wave discharges (SWDs) on the EEG. They occur mainly during quiet wakefulness, inattention and in the transition between sleep and waking (Guey et al., 1969). Absence epilepsy has a genetic predisposition without evidence of any structural lesion as its substrate (Niedermeyer, 1996). A thalamocortical dysfunction is assumed to play a major role in the underlying pathophysiology (Danober et al., 1998). Research on absence epilepsy is greatly facilitated by the use of animal models.

Both models discussed are validated models for spontaneous absence seizures in humans. The hallmark is the appearance of bilateral generalized SWDs on the electro-encephalogram (EEG) (Figure 3), which are of higher frequency than in man (7-12 Hz versus 3 Hz, respectively). In addition, absence seizures in rats appear later during life than in humans (during adulthood in rats versus childhood with tendency to disappear with adulthood in humans). However, in both animals and humans, absence seizures are believed to have the same pathophysiological background. The involvement of a thalamocortical circuit, particularly the contribution of the ventrobasal thalamus (VB) and the nucleus reticularis of the thalamus (NRT), in the propagation of absence seizures has been established for several years (Marescaux et al., 1992; Danober et al., 1998). Aberrant corticothalamic rhythms are believed to be the substrate of SWDs. Recently, it has been demonstrated that SWDs have a

focus in the peri-oral region of the somatosensory cortex from where they spread over the rest of the cortex (Meeren et al., 2002). This does not inevitably mean that the focus is also the source of the defect underlying SWDs (Crunelli and Leresche, 2002).

The *WAG/Rij* strain has been developed in Rijswijk (the Netherlands). The main difference between GAERS and the WAG/Rij strain is that the latter has two types of SWDs on the EEG. The type 2 SWD is not accompanied by behavioral signs and seems to be a more localized phenomenon (Midzianovskaia et al., 2001). The discharges in WAG/Rij rats are also less frequent, shorter in duration than in GAERS and can be accompanied with mild clinical manifestations (facial myoclonic jerks) (Coenen and Van Luijtelaar, 2003). The WAG/Rij rat will not be discussed any further as we chose to work with GAERS.

We preferred to utilize the *GAERS* model because they do not display the type 2 SWDs and GAERS have longer and more frequent SWDs, which makes them favorable to work with. The absences are characterized by behavioral arrest and are sometimes accompanied by rhythmic twitching of the vibrissae. Seizures develop gradually and the first SWDs appear around postnatal day 30. They are rare (1 or 2/hour), short lasting (0.5 to 3 s), with a low frequency (4 to 7 Hz) and variable morphology (Marescaux et al., 1992; Dedeurwaerdere et al., 2005). At the age of four months, 100% of the GAERS have developed SWDs on the EEG. Adult animals typically have 60 SWDs per hour, 0.5-75 s in duration and with a frequency of 7-12 Hz (Marescaux et al., 1992).

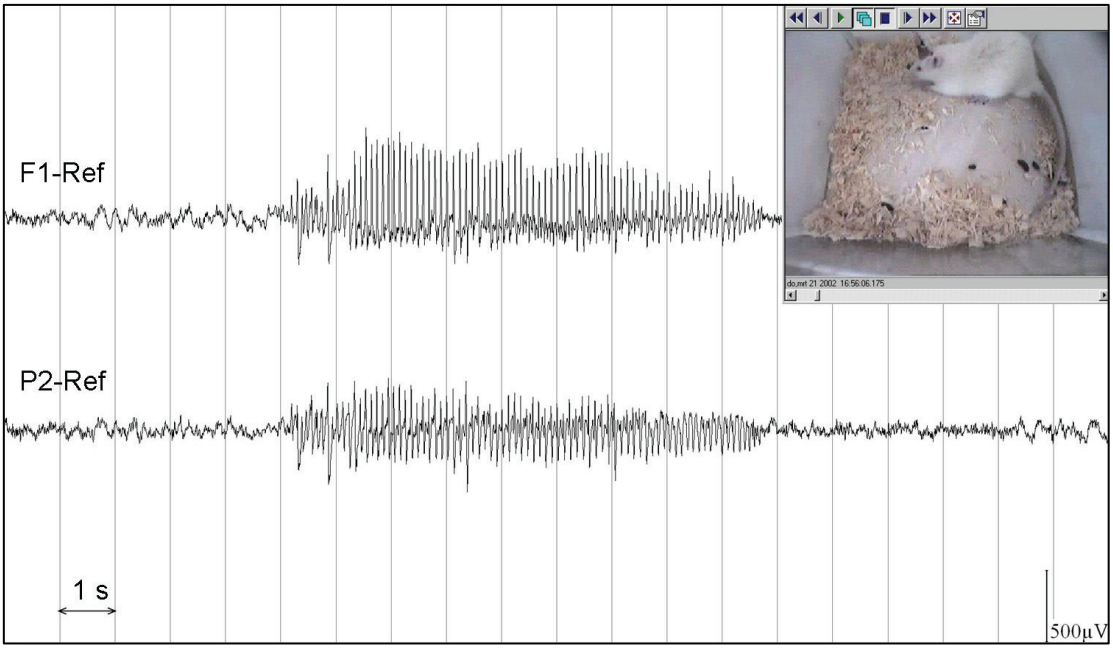

Figure 3: Bilateral generalized SWDs on the EEG in GAERS. Abbreviations: F1, frontal left; P2, parietal right; Ref, reference.

Drugs effective against absence seizures in humans (ethosuximide, valproate, trimethadione, benzodiazepines and topiramate) suppress SWDs dose-dependently, whereas drugs for convulsive or focal seizures (carbamazepine, phenytoin) are ineffective or aggravate absence seizures (Marescaux et al., 1992).

The frequent spontaneous seizures, the similarity with human absence epilepsy and the gradual development of epilepsy make this an attractive model to study epileptogenesis and treatments that can interfere with epilepsy. Another strong point is that SWDs appearing on the cortical EEG strictly correlate with the occurrence of the numerous clinical absences. Therefore, EEG recordings can be used to quantify the appearance of SWDs and absences.

1.3.2 Models of temporal lobe epilepsy (symptomatic epilepsy)

Of the various epilepsies and epilepsy syndromes the acquired epilepsies account for approximately 30-49% of the new cases (Herman, 2002). Acquired epilepsy is a consequence of brain-damaging insults such as: head trauma, stroke, brain infection, brain surgery or status epilepticus. Epileptic processes consist of three phases: the *initial insult* followed by the *latent period* (epileptogenesis) resulting in *recurrent seizures* (symptomatic epilepsy).
The models we are focusing on are most closely approximating human temporal lobe epilepsy (TLE). Temporal lobe epilepsy with complex partial seizures is in 70% of cases associated with hippocampal sclerosis as a pathologic substrate (Engel et al., 1999).

Ten percent of all acquired epilepsies in humans evolve after *status epilepticus* (SE) and the risk of developing epilepsy as a consequence of SE ranges between 30 and 80% (Shorvon, 2002; Herman, 2002). SE can be induced in animals by a number of different stimuli. In rodents, it is typically induced by kainic acid, pilocarpine or by sustained electrical stimulation of the amygdala, perforant path or hippocampus. Unlike kindling and other models of acquired epilepsy, a high proportion of the animals that survive the initial status develop spontaneous seizures after a latent period. Disadvantage is the high mortality during the SE and the aggressiveness of the survivors caused by destruction of parts of the limbic system. For the purpose of drug or other treatment efficacy studies, the status epilepticus model is laborious and time consuming, because the rats have to be continuously recorded for spontaneous seizures during several weeks or even months after the status (Loscher, 2002).

In the current study we used the electrical amygdala *kindling model.* Kindling is currently the most widely used animal model for epilepsy and can be performed in many species like rats, mice, cats, dogs, primates and even amphibians. Different brain structures (e.g. amygdala, hippocampus, piriform cortex) can be targeted electrically or chemically (e.g. glutamate, kainate). During the kindling process seizure severity and duration gradually progress. Its relatively slow onset due to daily or more frequent brief stimulation, allows detailed study of the events associated with the epileptogenic process. In addition, it offers the

advantage that seizures can be elicited at will. On the other hand these animals will not develop spontaneous seizures with traditional kindling protocols.

Because of the growing need for an animal model of complex partial seizures based on a genetic predisposition, two new lines of rats have been developed that are kindling-prone (Fast rats) or kindling-resistant (Slow rats) (Racine et al., 1999). These Fast-kindling and Slow-kindling rats are a parent mixture of two outbred strains that showed strong genetic control in the rate of amygdala kindling, with faster kindling rates in the Fast rats (Racine et al., 1999). Obvious differences between the two strains in cholinergic, monoaminergic and glutamatergic systems are not found (McIntyre et al., 2002). However, there are large strain differences in their response to GABA modulators. The Fast rats are very sensitive to negative modulators like bicuculline and picrotoxin and experience convulsive seizures to those drugs at much lower concentrations than the Slow rats (Racine et al., 2003). By contrast, the Slow rats are more sensitive to positive GABA modulators like pentobarbital and diazepam and experience sedation at doses that do not significantly affect the Fast rats (McIntyre et al., 1999). In agreement with this differential sensitivity to GABAergic agents, the pattern of $GABA_A$ subunit expression in the two strains is importantly different. Besides differences in excitability and epileptogenesis, the Fast strain also shows other natural differences with the Slow strain such as behavioral comorbidities including impulsivity, learning impairment, an attention deficit disorder and increased body weight during development (Anisman and McIntyre, 2002). Therefore, these Fast rats are of great interest to determine the therapeutic effects of VNS on memory and body weight next to kindling development and seizures.

1.4 Rationale and outline of the thesis

As outlined before, there is a continuous impetus to search for new and innovative treatment strategies. Neuromodulation is the science of how electrical, chemical and mechanical interventions can modulate or change central and peripheral nervous system functioning by initiating and influencing neurophysiological signals. Modulatory synapses in the central nervous system transmit information that will have long-lasting effects on the postsynaptic neuron's metabolic activity and on its response to subsequent input. These effects are fundamental to the development and adaptation of the nervous system and are believed to underlie higher functions such as learning and memory. Lately, neuromodulation has been defined by the Journal of the International Neuromodulation Society as "the therapeutic alteration of activity in the central, peripheral or autonomic nervous systems, electrically or pharmacologically, by means of implanted devices. Since, many neuromodulation therapies have been developed in several research fields, e.g. heart rate disorders, breathing disorders, movement disorders, pain, incontinence, spasticity, paralysis, depression and epilepsy. However, the definition of neuromodulation as "a form of therapy in

which neurophysiological signals are initiated or influenced with the intention of changing the function and performance of the nervous system to achieve therapeutic effects" covers this dissertation probably best.

The potential of such modulating and maybe even anti-epileptogenic therapies could be of great significance. They could slow down, alter processes underlying epilepsy or might prevent and even cure epilepsy. The current research project investigated the modulation capability of treatments (LEV and VNS) for patients with refractory epilepsy. Studies in man are hampered by the heterogeneity of patient populations (age, course of the epilepsy, type of epilepsy, AED regime and genetic background) and the difficulty to study therapy-related effects in a systematic way. Therefore, investigation was performed utilizing two models mimicking epilepsy in humans. They are both chronic models with seizures evolving from true and genetically-driven epileptogenesis. GAERS have inborn epilepsy and Fast rats have a genetically determined sensitivity for electrical amygdala kindling.

Levetiracetam (LEV) is a novel well tolerated AED approved as an adjunctive therapy for epilepsy patients with refractory partial seizures with or without secondary generalization. Anti-epileptogenic effects of LEV in addition to anti-epileptic effects have been reported in the rat amygdala kindling model for temporal lobe epilepsy (Loscher et al., 1998; Stratton et al., 2003) and the spontaneously epileptic rat (SER), a model of primary generalized epilepsy characterized by spontaneous tonic convulsions and absence seizures (Sasa et al., 2003). LEV suppresses kindling development at doses devoid of adverse effects with persistent reduction in afterdischarge duration after termination of treatment (Loscher et al., 1998). In the SER model study, only reported as an abstract, LEV was administered before the appearance of spontaneous seizures and was terminated at the expected age for seizure expression, which resulted in a lower seizure number in pre-treated animals (Sasa et al., 2003). These exciting findings have motivated us to evaluate the effect of chronic LEV administration early in life on the development of spontaneous SWDs in GAERS.

Vagus nerve stimulation (VNS) has been used since 1988 and at present approximately 30 000 patients are being treated with VNS worldwide. Experience and knowledge about VNS is rapidly increasing, however several questions remain unclear. VNS is used in generalized and partial epilepsy (Ben-Menachem, 2002), although responder groups have not been clearly identified. Currently 30% of patients treated with VNS will not benefit from VNS. Understanding VNS could improve seizure outcome by identifying specific epilepsy syndromes or types of epilepsy that respond well to VNS or by optimizing stimulation parameters. The precise MOA by which VNS exerts its anti-epileptic effect has not been elucidated yet. Through stimulation of the vagal afferent fibers in the neck, a large number of intracerebral structures are potentially affected. Initial animal studies with VNS showed promising results in reducing both ictal and interictal EEG abnormalities (Lockard et

al., 1990; Woodbury and Woodbury, 1990; Woodbury and Woodbury, 1991; Zabara, 1992; McLachlan, 1993; Takaya et al., 1996). These findings laid the foundation for further development of VNS as a treatment for human epilepsy. However, VNS efficacy in animals has primarily been assessed in acute models (3-mercaptopropionate, pentylenetetrazole, maximal electroshock, penicillin or strychnine application) utilizing application protocols immediately before and/or after seizure provocation. Only a few studies have assessed the effect of VNS in chronic animal models of epilepsy (Lockard et al., 1990; Munana et al., 2002), which is probably related to practical issues of chronic VNS in animals. It is clear that additional research is needed using chronic animal models and using both acute and chronic VNS protocols. Moreover, such fundamental data for idiopathic epilepsy are presently completely lacking. Information about the potential efficacy of VNS in GAERS, a validated animal model of absence epilepsy, could help to clarify the general principles that underlie VNS, although extensive therapeutic use of VNS in absence epilepsy is unlikely. In addition, we have investigated the effect of VNS on epileptogenesis and seizures in a model of partial epilepsy. We examined whether VNS could interfere with the development of amygdala kindling and whether seizures could be aborted in Fast rats.

Recently, VNS has been explored as a treatment for cognitive disorders and obesity (Sjogren et al., 2002; Kneedy-Cajem et al., 2002). Therefore, we also have performed more detailed research concerning effects on memory and body weight in the Fast rat strain, which has natural abnormalities like impaired learning and increased body weight.

Several functional imaging studies have been conducted to investigate the activation or inhibition of brain structures by VNS. These studies found changes on both sides of the brain by unilateral left VNS and pointed out a key role for the thalamus and medial temporal lobe structures in the MOA of VNS (Vonck et al., 2000; Van Laere et al., 2002; Chae et al., 2003). However, there is no consensus on other activated structures neither on the type of changes (inhibition or excitation). This discrepancy can be attributed to a number of confounding factors such as the differences between the imaging techniques used (PET, SPECT, fMRI), the contrast agents, scanning protocols, stimulation parameters, medication regimes, course of the illness and treatment response. Heterogeneity of the patient samples is difficult to avoid. In addition, data gathering from healthy subjects is impossible for ethical reasons as VNS is a relatively invasive procedure. Therefore, we have explored the possibility to investigate the effects of VNS on brain metabolism in rats using small animal PET.

The aim of this thesis was to investigate the effect of chemical (chronic treatment with LEV) and electrical (acute and chronic VNS) neuromodulation in chronic animal models of epilepsy. The following questions were raised:

1. Effects of chronic LEV treatment on seizure development

a) Do young GAERS have comparable SWDs (morphology, number, duration) as adult GAERS?
b) What is the effect of chronic LEV administration on SWDs in young GAERS?
c) Are SWDs in young GAERS altered after discontinuing chronic LEV treatment?
d) If SWDs are altered after discontinuing chronic LEV treatment, is this still the case when these animals reach adulthood?

2. Efficacy and spectrum of vagus nerve stimulation

a) Is it practically feasible to stimulate the vagus nerve of rats acutely and chronically?
b) Can home-made cuff-electrodes activate different vagal nerve fibers?
c) Does chronic VNS induce nerve damage?
d) Does VNS interrupt ongoing SWDs when applied immediately after their onset in a model of absence epilepsy?
e) Does acute VNS decrease SWDs in number and duration?
f) Does chronic VNS decrease SWDs in number and duration?
g) Does acute VNS interrupt generalized convulsions in rats?
h) Does chronic VNS have a prophylactic effect on generalized convulsions in rats?
i) Can chronic VNS interfere with epileptogenesis in rats?
j) Does VNS cause permanent changes in the epileptic network?
k) Does chronic VNS improve spatial memory in rats?
l) Does chronic VNS reduce body weight in rodent models of epilepsy?
m) What are the side-effects of chronic VNS in rats?

3. Mechanism of action of vagus nerve stimulation

a) Can small animal PET be used to detect changes in brain glucose metabolism induced by VNS?
b) Does acute VNS induce changes in glucose metabolism in rats?
c) Does chronic VNS induce changes in glucose metabolism in rats?
d) Do acute and chronic VNS induce different changes in glucose metabolism in rats?

Chapter 1 provides a general introduction on epilepsy, treatments for epilepsy and animal models for epilepsy. Also the rationale and the aim of this thesis have been outlined. Specific questions regarding neuromodulation and epilepsy are formulated.

Chapter 2 gives a general description of the methods and materials used in this animal study with emphasis on the cuff-electrode constructed for VNS in rats, including an electrophysiological and morphological evaluation.

Chapter 3 deals with the effect of chronic LEV treatment in GAERS. We have investigated the effect of LEV on the age-related SWDs in GAERS by chronic administration of LEV starting before the age of occurrence of SWDs. The effect of early chronic LEV administration on development of SWDs was evaluated in young GAERS during treatment, immediately after treatment termination and two months after the last LEV injection (age four months), when brain maturation was achieved and SWDs recorded on cortical EEG were numerous.

Chapter 4 describes the efficacy of acute and chronic vagus nerve stimulation in two animal models of epilepsy reported in three manuscripts. First, we investigated whether VNS applied at seizure onset can interrupt SWDs in GAERS. Subsequently, we determined whether SWD are suppressed or shortened in duration when VNS is applied several hours per day. Chronic VNS for one week was also evaluated in this validated model of absence epilepsy. Finally, we studied the effects of vagus nerve stimulation (VNS) on spatial memory, development of amygdala kindling, generalized convulsions and body weight using a Fast-kindling rat strain known to be prone to seizures and exhibiting several associated comorbid behaviors.

Chapter 5 focuses on the mechanism of action of VNS. The first manuscript is a review of the historical basis of VNS underlying the development of VNS for human use. In addition, animal and human research data on the mechanism of action of VNS are presented. The second manuscript was a pilot study to explore the potential of 2-[18F]-fluoro-2-deoxy-D-glucose (FDG) PET to investigate the effect of acute and chronic VNS on glucose metabolism in the brain of normal rats.

Chapter 6 discusses the results obtained from this thesis. The different observations from the experiments are related to each other and are discussed with regard to the current literature and the main questions and objectives. Future perspectives for further research within the field of neuromodulation for epilepsy are provided.

Finally a summary of the thesis is presented in English, Dutch and French.

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Chapter 2: General methodology

General methodology

This chapter consists of the general description of the methods used in the different experiments. For the purpose of the VNS experiments in rats we developed specific novel hardware such as simple cuff-electrodes and spiral silicone embedded cuff-electrodes. In the second part of this chapter, we describe an electrophysiological and morphological evaluation of the use of these electrodes.

2.1 Overview of the materials and methods

2.1.1 Animals

Wistar rats were purchased from a recognized breeder (Harlan, Netherlands). Genetic absence epilepsy rats from Strasbourg (GAERS) were bred and raised at the animal facility of Ghent University Hospital. All animals were treated according to guidelines approved by the European Ethics Committee (decree 86/609/CEE). The study protocols were approved by the Animal Experimental Ethical Committee of Ghent University Hospital. The Fast rats were bred and raised in the Life Science Animal Facility of Carleton University (Ottawa, CA). The experiments at Carleton University with the Fast rats were performed in accordance with the guidelines of the Canadian Council on Animal Care and a protocol approved by the Carleton University Animal Care Committee.

2.1.2 Housing

The animals were kept under environmentally controlled conditions (12 h light/dark cycles, lights on at 7 a.m., 20-23 °C, ± 50 % relative humidity) with standard food pellets (Pavan service PVBA, Carfil quality) and tap water ad libitum. Cages were provided with bedding and with additional nesting material for pregnant females or nursing mothers. The animals were housed in pairs in type III standard opaque plastic cages (22 cm x 32 cm x 19 cm, type III, Macrolon® Technilab-BMI). Operated adults were single housed in taller cages (Type III).

Figure 1: Experimental setup with glass square cages and flexible leads for acute experiments in rats.

When *acute experiments* (short-term experiments) were performed, the animals were moved to square glass cages (30 cm x 30 cm x 30 cm, Figure 1). The animals stayed at maximum 8 h in this acute setup. The electrodes on their heads were connected with a flexible lead to the EEG or the stimulation device (for vagus nerve stimulation or kindling). EEG was

acquired using a H2O™ portable digital 32-channel EEG-recorder (Telefactor Corporation, USA). Data were saved digitally on a dedicated PC.

In our laboratory, we also have a *long-term video-monitoring unit* for 16 rats (Figure 2). The cages were equipped with spring covered flexible leads (to avoid gnawing at the leads by the rat; Plastics One INC, USA), a spring (allows up and down movements of the rat) and a communicator or swivel (allows the rat to walk around in the cage without cables getting mixed up, Plastics One INC, USA). Leads (flat cables) were connecting the swivel with the EEG recorder. If required, video images could be simultaneously recorded with the EEG.

Figure 2: Video-EEG monitoring unit. A. Cage with flexible lead (a), a spring (b) and a swivel (c). B. Rats in video-EEG monitoring unit; a, flat cables to EEG recorder.

2.1.3 Surgical techniques

Before surgery, the surgical area was cleaned and disinfected with H.A.C. (Hospital antiseptic concentrate, chlorhexidine: 150 mg/l and cetrimidine: 1500 mg/l). Surgical instruments were sterilized by dry heat (30 min at 180 °C).

After a presurgical evaluation (vivid reaction to stimuli, healthy fur, bright eyes, not too skinny) to insure that the animal was not ill, the rat was anesthetized and shaved (Figure 3). The rats were anesthetized with ketamine/xylazine (80 mg/kg and 7.5 mg/kg respectively, i.p.). The ketamine/xylazine mix was diluted with saline (0.9 % NaCl) till an injection volume of 2 ml/kg was reached. Ketamine is a dissociative anesthetic with a probable action on NMDA receptors which results in analgesia. Xylazine is an α-2-adrenergic agonist, which stimulates α-2 adrenoceptors and causes sedation and analgesia by a decreased release in norepinephrine in the central nervous system (negative feedback system). During the operation, additional ketamine (5 mg/kg) was given when sensorial pain stimuli, by squeezing

the foot pad, were felt. An electric heat pad was provided to preserve the rat's body temperature during surgery. Then the surgical site (neck and head) was disinfected with Hibitane, ethanol and Iso-betadine (10% polyvidon iodine) from the centre of the site to the periphery.

Figure 3: Presurgical preparations: weighing, anesthetizing, shaving and surgical equipment.

Implantation of the vagus nerve stimulation-cuff-electrode

- Simple cuff-electrode

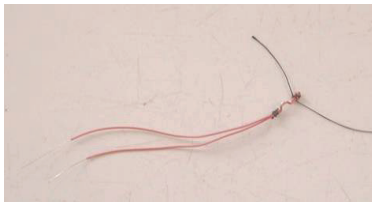

Figure 4: Simple cuff-electrode with lead wires.

The assembly consisted of two isolated stainless steel wires (bare/coated diameter= 0.2 mm/ 0.4 mm) and silk thread was used to hold the assembly together (Figure 4). The isolated wires were stripped on one side over a length of 3 mm and were fold in the shape of a cuff to hold the nerve. The stimulation poles were spaced out 1.5 mm from each other.

- Self-sizing spiral silicone cuff-electrode

The manufacture of the spiral silicone embedded cuff-electrode was adapted for small diameter cuffs (inner diameter= 0.8 mm), as previously described (Naples and Mortimer, 1988) and was also made in house (Figure 5). Two 1 x 3 mm dot contacts were cut in a 25-µm platinum foil (Alfa Aesar, USA) and the inclusion of contacts and leads was carried out according to Veraart et al. (1993). Electrode contacts were glued with a spiral silicone elastomer (MED-4210, Nusil Technology, CA) on a silicone rubber sheet (MED-4750 from Statice Santé, France with NuSil components). A second stretched sheet of silicone was then pressed on top of the first one. After curing of the silicone elastomer, rectangular windows (0.7 mm x 2 mm) were opened in the inner layer using the sharp section of a hypodermic needle. The electrodes were finally trimmed at 5 mm cuff-width.

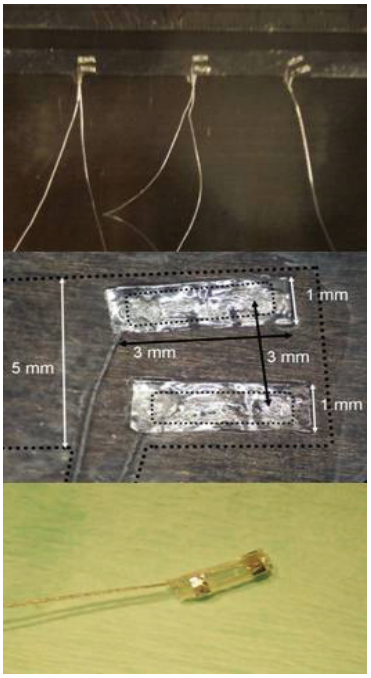

Figure 5: Fabrication of the spiral cuff-electrodes. Upper panel: three electrodes are manufactured on one silicone sheet and are cut above and below the electrode contacts at 5 mm cuff-width. Middle panel: magnification of the electrode contacts with indication of the measurements; the centers of the electrode contacts (rectangular window of 0.7 mm x 2 mm) are spaced out 3 mm. Lower panel: finished spiral cuff-electrode with flexible wires.

- Cuff-electrode implantation

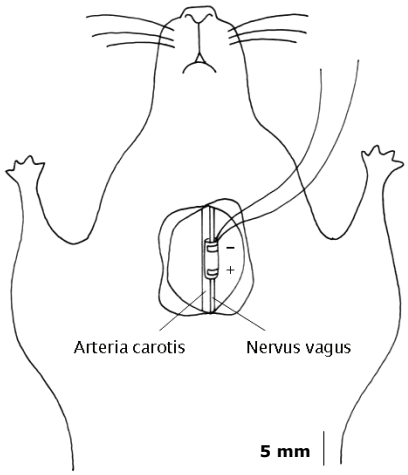

Figure 6: Positioning of the VNS cuff-electrode. The cuff-electrode is implanted in the cervical region around the left vagus nerve, which is located next to the arteria carotis. The cathode and anode are indicated on the picture as '−' and '+', respectively. The electrode wires are later on subcutaneously tunneled towards the head of the rat.

An overview of the procedure to implant a cuff-electrode around the vagus nerve is presented in figure 8.

The rat was fixated with hooks or tape on the sterile operating field. An incision (1.5 cm) was made over the left ventral region of the neck to allow free dissection of the left vagus nerve. The subcutaneous tissues were incised and blunt dissection was extended through the muscle layers. The left vagus nerve was isolated from connective tissue and the arteria carotis. Subsequently, the cuff-electrode was placed around the vagus nerve (Figure 6). The cuff-electrode was sutured in place to prevent displacement by animal movement and to secure the connection between the nerve and electrode. The wires were bended in a strain relief loop and sutured to the surrounding tissue (muscles). In this way, potential tension on the cuff-electrode due to animal movements (e.g. neck stretching) is avoided. The ends of the cuff-electrode were tunneled subcutaneously using a shunt-passing tool from the ventral cervical incision via the dorsal cervical region to the head, where they were fixated with acrylic cement together with the EEG electrodes.

Implantation of the EEG electrodes

The animal was fixated in the stereotaxic frame by means of ear bars (Figure 9 and 11). A midline incision was made on the head of the rat. After exposure of the skull, dependent on the number of epidural EEG electrodes small holes were bored for the positioning of the screws (Figure 11).

Epidural electrodes (Figure 7), consisting of a stainless steel screw attached to an isolated silver wire, were positioned on the skull opposite to the frontal cortex (2 mm anterior to bregma and 1.2 mm lateral to the midline) and were placed on the parietal cortex (2 mm posterior to bregma and 1.2 mm lateral to the midline). An epidural reference electrode was screwed at lambda.

Figure 7: Epidural electrode made of a stainless steel screw, a silver wire and a connection piece. Magnification 2x.

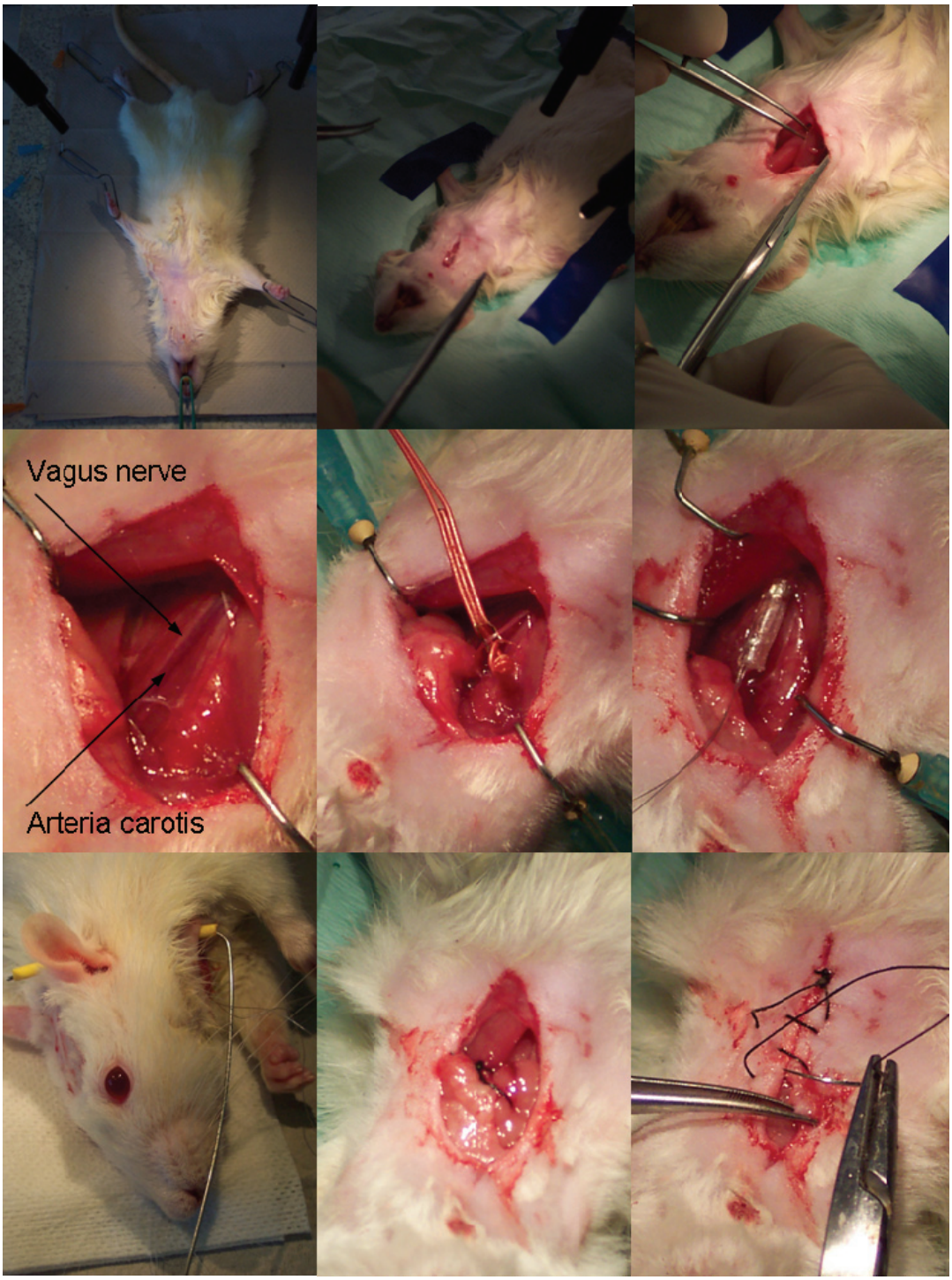

Figure 8: Overview of the implant procedure of two types of cuff-electrodes around the left vagus nerve. Upper panel: fixation of the rat on the operation field, midline incision, blunt dissection of the muscles; middle panel: isolation of the left vagus nerve from the arteria carotis and connective tissue, implantation of the simple cuff-electrode, implantation of the silicone cuff-electrode; lower panel: tunnelling of the lead wires subcutaneously towards the head, fixation of the electrode underneath the muscles and suturing of the incision wound.

Figure 9: Operation field with the rat fixated in the stereotaxic frame.

In case depth electrodes were required for amygdala kindling, bipolar stimulating/recording electrodes were implanted in both amygdalae (Figure 10 and 11). These home-made electrodes consisted of two twisted strands of 0.127 mm diameter Diamel-insulated Nichrome wire and were cut at approximately 1 cm. Coordinates for the implantation were 2 mm posterior to bregma, 4.5 mm lateral to the midline and 8.4 mm below the skull surface (Paxinos and Watson, 1998).

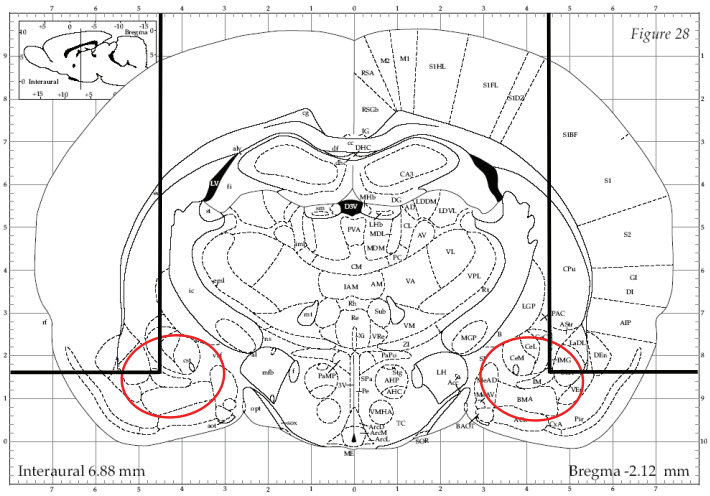

Figure 10: Amygdala placement of the bipolar electrodes. Red lines encircle the amygdalair nuclei.

In addition, five stainless steel screws, embedded in the skull were used to anchor the electrodes in place. The electrodes and screws were secured in place by dental acrylic (Figure 11).

Following surgery, the rats were given acetaminophen rectal gel, a topical analgesic (xylocaine gel, 0.2%) at the wound edges and a subcutaneous injection of saline (NaCl 0.9%, 2 ml/kg) and were then placed in plastic cages under warming lamps to maintain normal body temperature until they became behaviorally active again. The recovering animals were closely observed until they were breathing normally and were able to move around easily. The rats were then returned to the colony room and allowed up to two weeks of recovery before the beginning of the recordings.

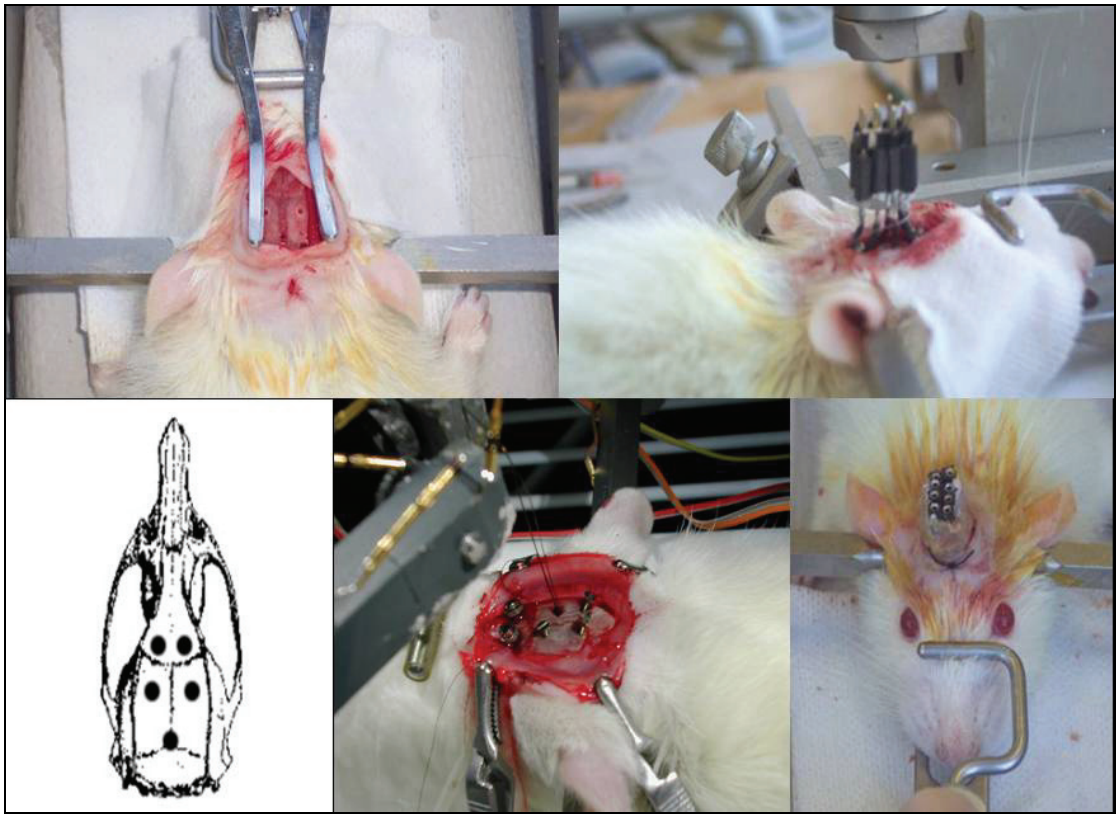

Figure 11: Implantation of EEG electrodes. Upper panel: fixation of the rat with ear bars in the stereotaxic frame and preparation of the skull, placement of the epidural EEG electrodes; lower panel: position of epidural screw electrodes, implantation of the depth stimulating/recording electrodes and fixation of the electrodes with acrylic cement.

2.1.4 Kindling procedures

The kindling model is currently the most widely used animal model for temporal lobe epilepsy. During the kindling process seizure severity and duration gradually progress. It offers the advantage that seizures can be elicited at will and that it allows detailed study of the events associated with the epileptogenic process.

Afterdischarge threshold determination

Two weeks after surgery, initial (prekindling) afterdischarge thresholds (ADTs) were determined for both amygdalae. The ADT was defined as the minimum stimulus intensity necessary to provoke a clearly discriminable, high-voltage, electrographic seizure event (an AD, Figure 12) that outlasted the stimulus by two or more seconds (Kelly et al., 1999). To assess the threshold a 1 s, 500 µs pulse duration, 60 Hz square wave stimulus of progressively increasing intensity (15, 25, 35, 50, 75, 100, 150, 200, 300, 400, 500, 600 µA) was delivered using a constant current generator until an AD was triggered. The interval between stimulations was 1 min. Post-kindling ADTs were determined the day after kindling was completed.

Kindling

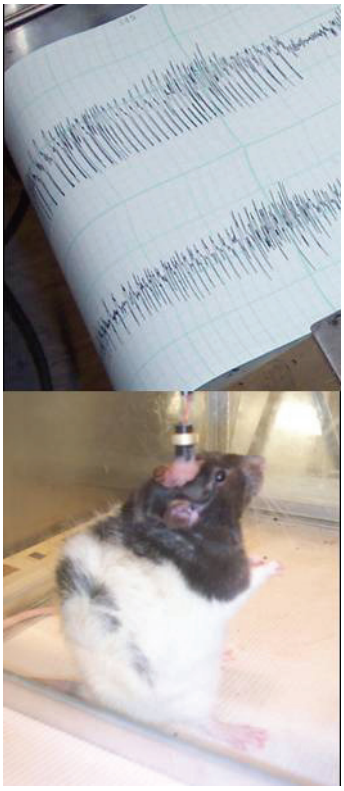

Figure 12: AD on the EEG and behavioral stage-5 seizure (characterized by generalized convulsions with rearing and falling) evoked by kindling.

Twenty-four hours after the ADT determination, kindling began. All rats were stimulated in the amygdala once daily at their individual ADT intensity until five stage-5 generalized convulsive seizures, characterized by forelimb clonus with rearing and falling, had occurred (Racine, 1972). During the course of kindling, if the rat failed to respond with an AD, the rat was stimulated at a higher intensity until an AD was evoked (using the increment technique described at ADT determination).

The typical behavior associated with the initial amygdala ADs is behavioral arrest. With repeated stimulations, duration and severity of seizures gradually progress. Seizures are usually classified according to Racine (1972): stage 1, immobility, eye closure, twitching of vibrissae, sniffing, facial clonus; stage 2, head nodding associated with more severe facial clonus; stage 3, clonus of one forelimb; stage 4, rearing, often accompanied by bilateral forelimb clonus; stage 5, rearing with loss of balance and falling accompanied by generalized clonic seizures. Stage-5 seizures represent the final seizure stage (Figure 12). Typically between 10 and 15 stimulation trials are required before a stage-5 seizure is successfully triggered from an amygdala focus. The exact rate of amygdala kindling is dependent not only on the strain of rat, but also on the particular amygdala nucleus in which the stimulation electrode is situated.

2.1.5 Vagus nerve stimulation

Vagus nerve stimulation (VNS) was performed by connecting the cuff-electrode to an external NeuroCybernetic Prosthesis device (NCP, model 100; Cyberonics Inc., USA). As in patients, output current was ramped up to a just tolerable level of stimulation (Handforth et al., 1998). When a stimulus of higher intensity was given, animals behaviorally reacted by flattening the ears (Dedeurwaerdere et al., 2004) and a fixed posture. The following stimulation parameters were used: pulse duration 500 μs and frequency 30 Hz. Output current was dependent on the type of cuff-electrode and the tolerable level was 1.5 mA for the simple cuff-electrode and 0.5 mA for the spiral silicone cuff-electrode.

A short test stimulus was delivered daily in treated animals to confirm that true stimulation had been delivered by evoking a behavioral response and to check the impedance and integrity of the electrode-to-vagus nerve interface (using the Lead Test of the NCP

programming software). The impedance values were always within the attainable range to deliver the desired output current by the pulse generator. However, the simple cuff-electrode had lower impedances than the spiral cuff-electrode. Factors that can influence the impedance are measuring aspects such as frequency, pulse duration or difference in voltage applied. As these parameters were the same during the impedance measurements of both electrodes numerous other factors which can influence impedance should be considered. The simple cuff-electrode has stainless steel contacts whereas the spiral cuff has platinum contacts. Stainless steel can initially result in lower impedance. Indeed, these electrodes first have lower impedance values, however, after prolonged stimulation of a few days up to 2 weeks impedance increased dramatically. In addition, a closer contact of the simple cuff with surrounding fluid could also contribute to the initial lower impedance.

2.1.6 EEG analysis

For the analysis of the spike and wave discharges (SWDs) (Figure 13, A) on the EEG of adult GAERS, a previously described and validated detection software package was used (Van Hese et al., 2003). Once a list of marked time instances was obtained by the detection method, a number of post-processing steps were performed by the software package: detections lasting less than 0.5 s were discarded and two detections less than 1 s apart were taken together. SWDs detected by the software package were visually confirmed. In these animals, both the number per hour and mean duration of the SWDs during the recording hours were calculated as well as the cumulative duration of the SWDs per hour (summation of the duration of all SWDs per hour) as a quantitative measure for seizure evaluation.

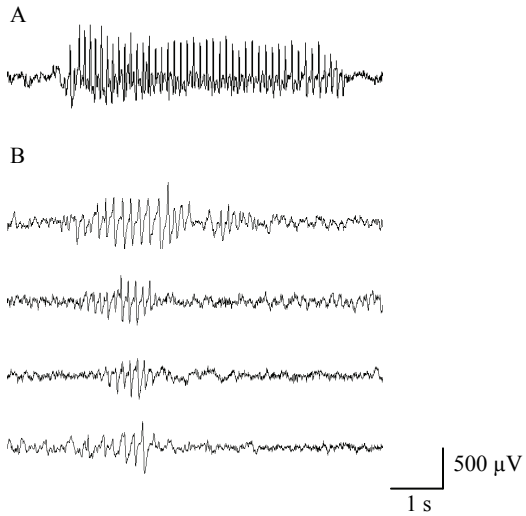

Figure 13: Different types of SWDs. A. Typical adult SWD (amplitude: >3 times baseline amplitude; peak frequency: 7-12 Hz; duration > 0.5 s); B. SWD-like events i.e. SISWDs in young GAERS (PN57-PN64) consist of irregular short discharges, with clear spike and wave aspects characterized by lower amplitude (< 3 times baseline amplitude) or lower peak frequency (5-7 Hz).

GAERS (under the age of 4 months) may not all have developed typical SWDs yet or have infrequent SWDs (Marescaux et al., 1992). For quantification purposes, epileptiform events were classified into two closely related types, because the morphology of SWDs in young GAERS was found to be less pronounced and more variable when compared with SWD patterns in adult GAERS (Figure 13): i) typical SWDs also

present in adult GAERS (*amplitude*: >3 times baseline; *peak frequency*: 7-12 Hz; *duration* > 0.5 s) and ii) SWD-like activity, which we have termed 'short irregular spike and wave discharges' (SISWDs), with clear spike and wave aspects characterized by lower amplitude (< 3 times baseline amplitude) or lower peak frequency (5-7 Hz) and shorter in duration (< 2 s).

The software package specifically developed for the detection of SWDs was not appropriate for the scoring of the more irregular variant of the SWDs (SISWDs). Hence, epileptiform events in young GAERS (PN57-PN64) were quantified by means of visual inspection of the EEG using TWIN™ off-line EEG analysis (Telefactor Corporation, USA) by an experienced electroencephalographer (SD).

2.1.7 Histology

In the electrical kindling experiments, brains were collected for electrode placement verification. The rats were perfused 24 h after the experiments. Each rat was deeply anesthetized with 65 mg/kg sodium pentobarbital and was then perfused intracardially with saline followed by 10% formalin. One day later, the electrodes were removed from the cranium and the brains were excised and stored in 30% sucrose for at least three days before sectioning. Frozen sections of 40 µm were taken through the electrode tract tips and stained with cresyl violet to identify the kindled sites (Figure 14).

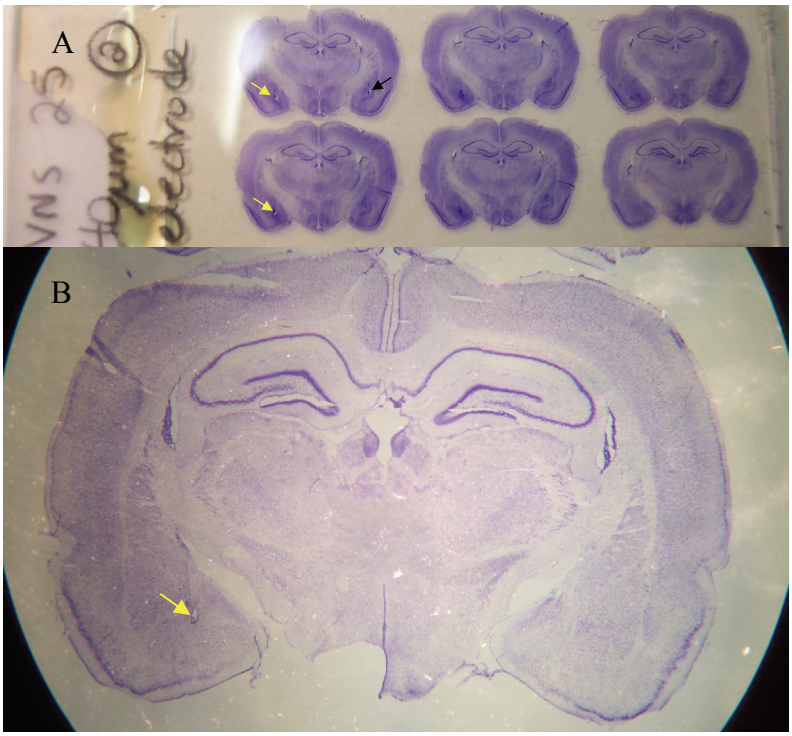

Figure 14: A. Serial coronal slices (40 µm) of the rat brain stained with cresyl violet. The electrode tract tips can be followed on the different slices. B. Magnification of coronal slice with electrode placement in left amygdala. Electrode placements are indicated with an arrow.

2.2 Electrophysiological and morphological evaluation of the use of two cuff-electrodes for vagus nerve stimulation in rats.

This study has been presented as a poster at the 26th International Epilepsy Conference (Paris, France, 2005) and was published in abstract form in Epilepsia. It was beyond the scope of this Ph.D. thesis to develop a new cuff-electrode stimulation interface or to perform a detailed physiological and morphometrical study on cuff-electrodes. Hence, the aim was to evaluate two types of ready-designed cuff-electrodes fabricated in house on an electrophysiological and morphological basis for their use in stimulating the vagus nerve of rats on an acute and chronic basis.

2.2.1 Abstract

Purpose: A better understanding of the mechanism of action of vagus nerve stimulation (VNS) could lead to stimulation parameter optimization and identification of responder groups. Animal research is therefore crucial. The aim of this study was to evaluate two types of stimulation cuff-electrodes for rat nerves on electrophysiological and morphological basis.

Methods: Two cuff-electrodes were evaluated: i) a simple cuff-type electrode and ii) a self-sizing spiral silicone cuff-electrode.

Electrophysiological measurements of compound nerve potentials induced by the two cuff-electrodes were performed under deep xylazine/ketamine anesthesia. Subsequently, several combinations of stimulation parameters (amplitude: 50-1500 μA, pulse duration: 50-500 μs) were tested.

For the evaluation of morphological modifications, vagus nerves were dissected from non-implanted control animals (n= 6). The simple cuff and spiral silicone cuff-electrodes were implanted around the left vagus nerve of respectively 27 and 10 rats and vagus nerves were dissected after 34 to 377 days of implantation. Nerve tissue was fixated with formalin, embedded in paraffin and subsequently stained.

Results: Both cuff-electrodes were able to elicit A, B and C fiber compound action potentials. The spiral silicone cuff-electrode required lower stimulus currents to trigger the different fibers.

Long-term simple cuff-electrode implantation caused substantial nerve damage, which was mainly mechanical in nature in contrast to the spiral silicone cuff-electrode, which induced significantly less morphological changes ($p < 0.05$).

Conclusion: Both cuff-electrodes were able to induce vagus nerve compound action potentials, but the spiral silicone cuff-electrode required lower output current. For long-term implantation, the spiral silicone cuff-electrode induced less morphological changes than the simple cuff-type electrode and was therefore recommended.

2.2.2 Electrophysiological evaluation of two cuff-electrodes

During our animal studies on VNS, we have used two types of cuff-electrodes, which were fabricated in house. The first electrode was a simple cuff-type electrode for acute experiments, which was easy to manufacture and inexpensive (Figure 4). The second electrode was a more sophisticated self-sizing spiral silicone cuff-electrode developed for long-term use, which took more time and specific equipment to manufacture and was more expensive (Figure 5).

We have tested the capability of both electrodes to induce compound action potentials (CAPs) in the vagus nerve. Electrophysiological measurements of compound nerve potentials induced by the two cuff-electrodes were performed under ketamine/xylazine anesthesia (80 mg/kg and 7.5 mg/kg respectively, i.p.). Firstly, the measurements of nerve potentials evoked by the simple cuff-electrode were performed at the Center of Neuroscience, University of Amsterdam (Amsterdam, the Netherlands). We have recently started to use the more sophisticated spiral silicone embedded cuff-electrode. Testing of this electrode took place at the Neural Rehabilitation Engineering Laboratory, Université Catholique de Louvain, (Brussels, Belgium) were this electrode was developed. During this electrophysiological evaluation, two spiral silicone cuff-electrodes were placed around the vagus nerve, the caudal one was used to stimulate and the more rostrally positioned electrode to record the evoked CAPs. The experimental setup is shown in figure 15. It was possible to measure the three fiber components (A, B and C) of the vagus nerve (Figure 15). Both cuff-electrodes were able to induce CAPs in the vagus nerve.

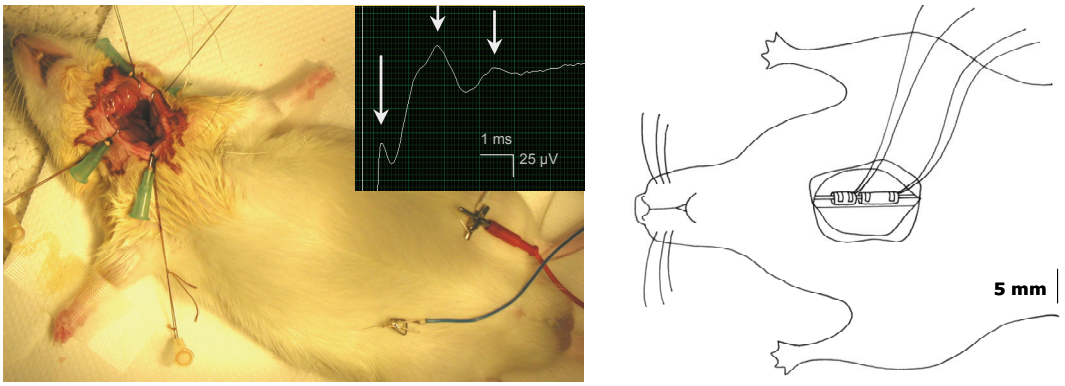

Figure 15: Overview of the setup for vagus nerve stimulation and recording with the self-sizing spiral silicone cuff-electrode. CAPs of A, B and C fibers of the vagus nerve evoked at output current 1000 µA and pulse duration 50 µs using the silicone cuff-electrode. The response of the A fibers appeared first and was partially enclosed by the stimulation artifact. Subsequently, the CAPs of the slower B and C fibers could be measured. Distance between cathode and registration electrode was 3.6 mm.

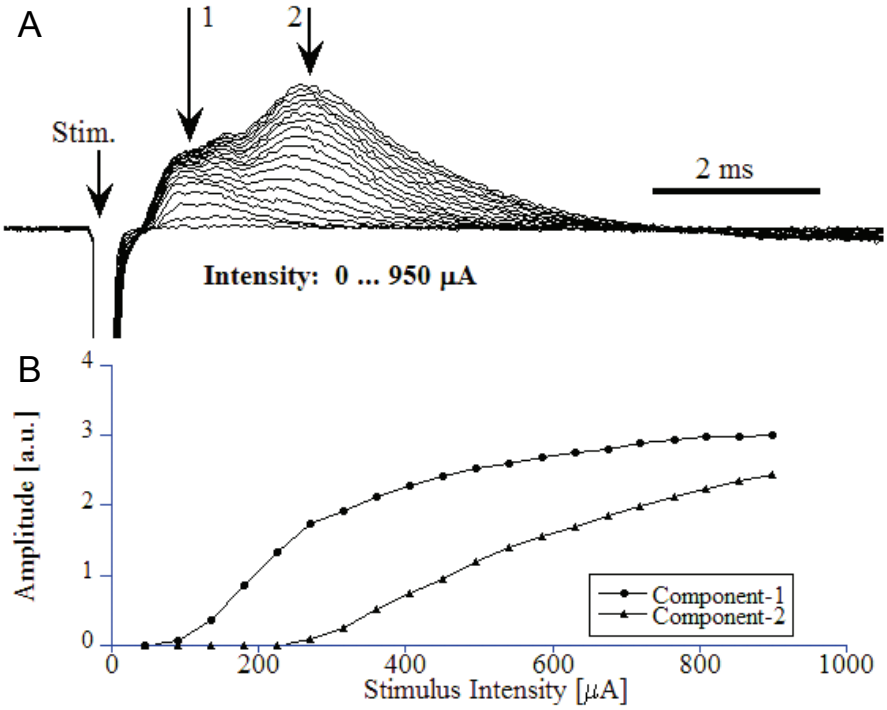

Figure 16: CAPs recorded after stimulation of the vagus nerve with the simple cuff. A. Input/output curve of CAPs recorded from the vagus nerve evoked by increasing current (0-950 µA) at pulse duration 200 µs using the simple cuff-electrode. B. The amplitude of the CAPs increased as a function of the stimulation intensity. A fibers (1) have a lower threshold than B fibers (2) and the two responses are clearly discriminable. C fibres activation was not present at these stimulation parameters. Distance between cathode and registration electrode (Teflon coated wire with isolation removed from the tip, 80 µm diameter, California Fine Wire, Canada) was 10 mm. a.u., arbitrary unit.

The amplitude of the CAPs increased when stimulation intensity was augmented (Figure 16). Threshold current values to excite large myelinated fibers (A and B fibers) are often low (15-70 µA; 100-200 µs) (Fenik et al., 2001). We found relative low stimulation thresholds for myelinated A and B fibers with both electrode types. To activate the C fibers higher current was needed. However, it has been argued that the activation of these C fibers is not required for the anti-epileptic effect of VNS (Krahl et al., 2001). The spiral silicone cuff-electrode required lower stimulus currents to trigger the different fibers than the simple cuff-electrode. Indeed, thresholds to elicit side effects were lower in the spiral silicone electrode than in the simple cuff (0.5 mA versus 1.5 mA). Most likely, the nerve is better isolated in the spiral silicone cuff, which prevents current to leak to the surrounding tissue.

2.2.3 Morphological evaluation of two cuff-electrodes

The left vagus nerve was sampled in 35 implanted animals and six control non-implanted animals for histological evaluation. At the conclusion of the implant period, the animals are brought under a deep level of anesthesia (overdose pentobarbital; 180 mg/kg, i.p.); this is a pain-free and stress-free death. Nerve tissue were resected and subsequently fixated with formalin and embedded in paraffin. Longitudinal sections were taken for examination of the nerve tissue and external epineurium. Thick sections (10 µm) were cut, mounted on slides, stained with hematoxylin and eosin (cells and nuclei), s100 (calcium binding proteins present in nerves) or myelin basic protein (mbp, staining of myelin) and coverslipped (Figure 17).

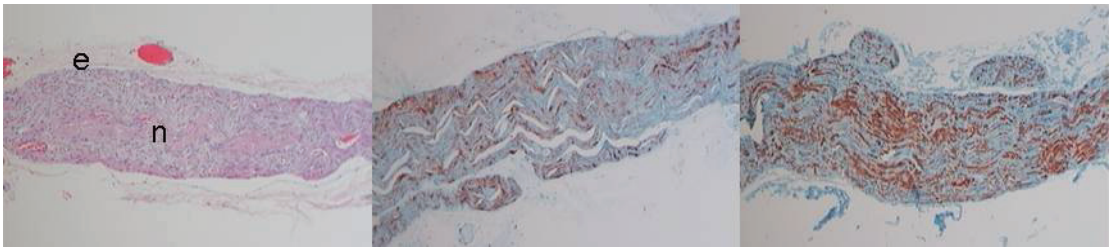

Figure 17: Staining of nerve tissue with hematoxylin/eosin, s100 and mbp (left to right respectively) of a normal nerve. Abbreviations: e, external epineurium; n, nerve.

Long-term implantation of simple cuff-electrode caused substantial nerve damage, which was mainly mechanical in nature in contrast to the spiral silicone cuff-electrode, which induced less morphological changes (Dedeurwaerdere et al., 2005).

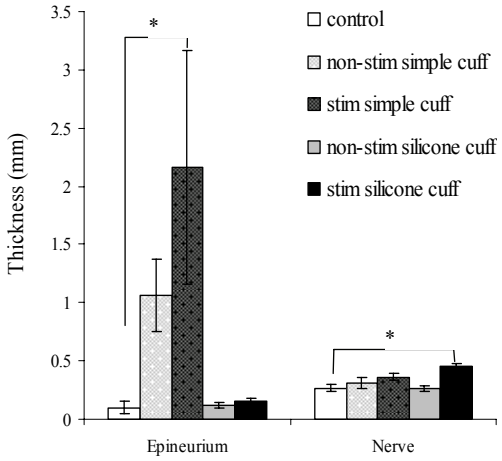

Figure 18: Effect of electrical stimulation and cuff implantation on epineurium and nerve thickness of the vagus nerve in rats. Abbreviations: non-stim, non-stimulated; stim, stimulated; data are expressed as mean ± SEM, significance is set at $P< 0.05$, * significantly higher than control nerve.

The thickness of the external epineurium was significantly increased in the stimulated simple cuff group when compared to the right intact (non-implanted) nerve of the animals ($p< 0.01$, Wilcoxon signed rank test) (Figure 18). Such a trend was also noticeable in the non-stimulated simple cuff group, but not in the spiral silicone cuff group. This is not surprising because the simple cuff-electrode consists of rigid leads, which may have caused friction with the nerve. Relative movement between cuff or cuff-leads and underlying nerve may have induced a continued proliferation of connective tissue, which could have led to the increase in thickness of the epineurium.

The thickness of the vagus nerve was significantly higher in the stimulated spiral silicone-cuff group when compared to the control group ($p< 0.05$, Mann-Whitney U test) and to the non-stimulated spiral silicone cuff group ($p< 0.05$, Mann-Whitney U test) (Figure 18). This was not the case for nerves stimulated through the simple cuff-electrode. Increase in thickness of the internal epineurium might be a possible cause. However, it is difficult to explain why this is only present in the stimulated spiral silicone group and not in the simple electrode group.

The epineurial sheath included blood vessels, sometimes foreign object reaction and fibrosis. Blood vessels were significantly increased in the non-stimulated ($p< 0.05$, Wilcoxon signed rank test) and stimulated ($p< 0.01$, Wilcoxon signed rank test) simple cuff group when compared to their corresponding intact right nerves. Fibrosis was increased significantly in the stimulated simple cuff group only. Electrical stimulation is considered in itself to be a conspicuous element of potential nerve injury. Prolonged (> 8h), high-frequency (> 50 Hz) electrical stimulation of a peripheral nerve induces neural injury (Agnew et al., 1999). Neural damage due to electrical stimulation is decreased or abolished by reduction of the frequency of stimulation, by stimulating at 20 Hz (versus 50 Hz) or by the use of an intermittent duty cycle (Agnew and McCreery, 1990; McCreery et al., 1995). In addition, it has been suggested that continuous electrical stimulation of peripheral nerves at a low frequency induces little or no neural damage, even if the stimulus amplitude is very high (McCreery et al., 1995). Based on these findings, it has been established that VNS should not exceed 30 Hz and continuous stimulation protocols should be avoided (Agnew and McCreery, 1990). In our studies, we bore these guidelines in mind and VNS was applied at 30 Hz. In the spiral silicone cuff-electrode there was no stimulation induced fibrosis. Hence, the increased fibrosis in the stimulation simple cuff-group is likely due to a combination of the rigid simple cuff and stimulation rather than to stimulation alone. Also inflammation was significantly increased ($p< 0.025$, Wilcoxon signed rank test) in the epineurium of the simple cuff groups. Improving the quality of the fabrication environment should reduce the chronic inflammation element of this immunological response.

In the spiral silicone cuff groups, components of the epineurial sheath were not altered. This is in line with a previous morphometrical study stating that the effects produced by the chronic implant of this spiral silicone cuff-electrode were negligible (Romero et al., 2001). The spiral silicone cuff-electrode has been manufactured under a laminar flow bench in a clean room. This is possibly the reason why significant inflammation of the vagus nerve was not present in the animals implanted with the spiral silicone cuff-electrode.

Transverse sections of samples from the spiral silicone cuff-electrode were made for electron microscopy (Figure 19). Appearance of the myelinated axons was not different between non-stimulated and stimulated groups. However, in both groups, nerves were found

with decreased myelination of axons. Previous studies were unable to establish a link between morphological abnormalities caused by chronic cuff implantation and functional deficit (Grill and Mortimer, 2000). Epineurial electrodes have been found to induce axonal degeneration, however, the pathology had no apparent effect on the functional response to electrical stimulation (Koller et al., 1992).

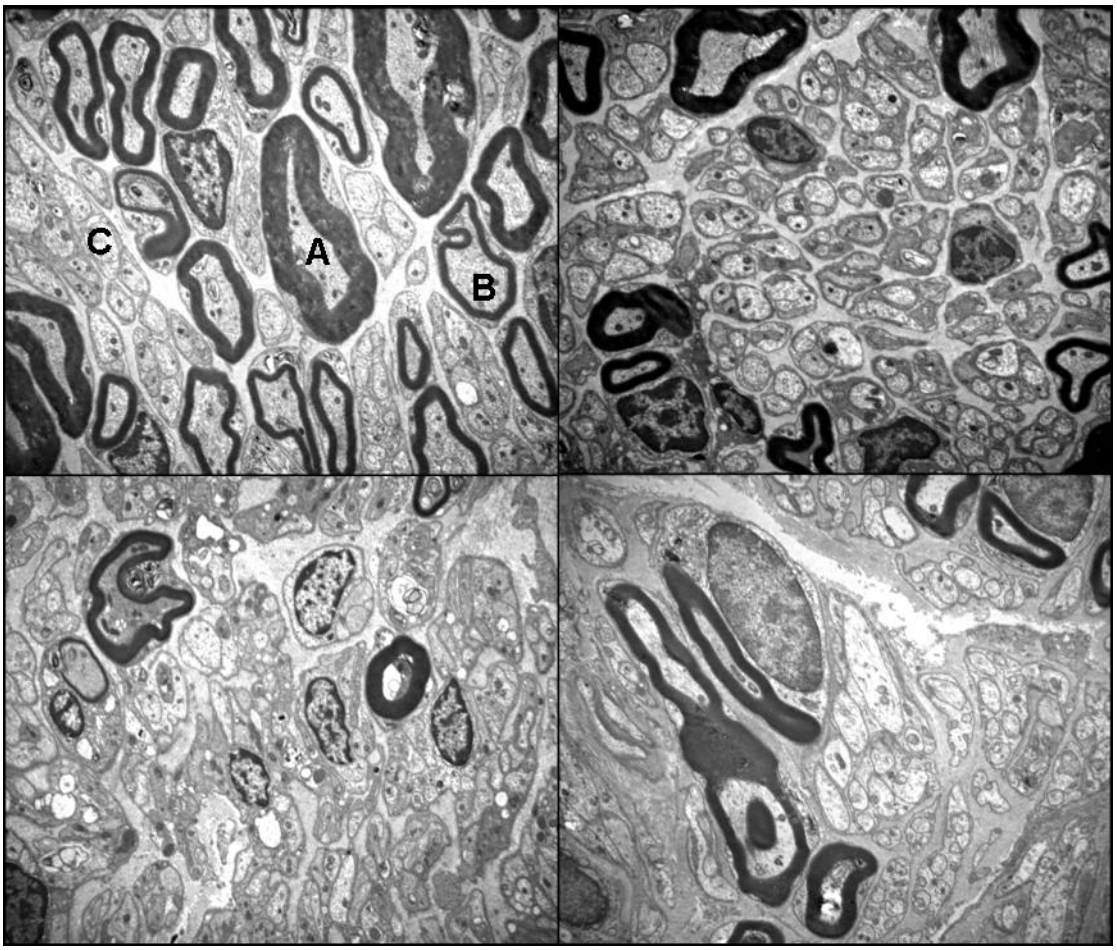

Figure 19: Electron microscopy of the myelination of the vagus nerve. Upper panel: non-implanted nerves, lower panel: non-stimulated implanted nerve with decreased myelination (left) and stimulated nerve with almost normal myelination (left). Abbreviations: A, A fiber; B, B fiber and C, C fiber.

The simple cuff-electrode was a handy electrode for short term experiments. It was easy to fabricate, to implant and was assembled with inexpensive materials. For long-term implantation, the spiral silicone cuff-electrode was recommended, because of its more flexible cabling and contacts it induced less morphological changes than the simple cuff-type. In addition, less current is required to activate the different fibers, which is preferred for the battery life.

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Chapter 3: Efficacy of levetiracetam

Seizure (2005) 14, 403–411

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Chronic levetiracetam treatment early in life decreases epileptiform events in young GAERS, but does not prevent the expression of spike and wave discharges during adulthood

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Purpose: In genetic absence epilepsy rats from Strasbourg (GAERS) age-related absence seizures start to appear from postnatal day (PN) 30 concomitant with 'spike and wave discharges' (SWDs) appearing on cortical EEG recordings. The aim of this study was to investigate the effect of early chronic levetiracetam (LEV) treatment on the development of SWDs in young and adult GAERS.
Methods: From PN23 until PN60, LEV (54 mg/kg, i.p.) was administered once daily to GAERS (n= 8), while control GAERS (n= 7) received saline (0.9% NaCl, i.p.). All animals were implanted with four epidural EEG electrodes at PN51. EEG was recorded for 3 h daily, during the last four days of the treatment (PN57-PN60) and during four additional days after treatment had been terminated (PN61-PN64). The animals were monitored again at the age of four months (PN120-PN124), about two months after the last administration of LEV.
Results: During treatment, epileptiform events in the LEV group were significantly reduced (62%, $p < 0.05$) in comparison with the control group. During the following four days, epileptiform events were reduced in the LEV group, with an average difference of 53% ($p = 0.064$). Once the animals had reached adult age, there was no difference in epileptiform events between the LEV group and controls.
Conclusion: In this study, chronic LEV administration induced a reduction in epileptiform events in young GAERS. This effect persisted in some extent after treatment cessation (PN61-PN64), which might indicate a slowing down of epileptogenic processes. However, at the age of four months all animals revealed a similar expression of epileptiform discharges.

Key words: levetiracetam, anti-epileptogenic, absence epilepsy, GAERS, spike and wave discharges (SWDs)

1. Introduction

Every year the incidence of new epilepsy cases is between 26 and 70 per 100 000 (Hauser, 1998). Despite the development of new pharmacological treatments, approximately one third of epilepsy patients does not respond to anti-epileptic drugs (AEDs), and are referred to as refractory patients (Kwan and Brodie, 2000). Treatments with anti-epileptogenic potential could modulate the processes underlying the development of epilepsy. Anti-epileptogenic properties have been attributed to some AEDs like valproate (Silver et al., 1991), lamotrigine (Stratton et al., 2003), and pregabalin (Andre et al., 2003) as well as to vagus nerve stimulation (Fernandez-Guardiola et al., 1999). Anti-epileptogenic effects of levetiracetam (LEV) in addition to anti-epileptic effects have been reported to appear in the rat amygdala kindling model for temporal lobe epilepsy (Loscher et al., 1998; Stratton et al., 2003) and the spontaneously epileptic rat (SER), a model of primary generalized epilepsy (Sasa et al., 2003).

LEV is a novel well tolerated AED approved as an adjunctive therapy for epilepsy patients with refractory partial seizures with or without secondary generalization (Cereghino et al., 2000). LEV has an anti-epileptic effect in a broad range of animal models mimicking both partial (Loscher and Honack, 1993; Loscher et al., 2000; Glien et al., 2002) and generalized epilepsy in man (Loscher and Honack, 1993) including genetic absence epilepsy rats from Strasbourg (GAERS) (Gower et al., 1995). In contrast, LEV revealed no effect in the two conventional screening tests, namely the maximal electroshock and pentylenetetrazole models (Loscher and Honack, 1993). The mechanism of action of LEV differs from other AEDs and is as yet not fully elucidated.

Studies conducted on AEDs administered during the process of epileptogenesis have so far focused on animal models of acquired symptomatic epilepsy (Pitkanen, 2002; Andre et al., 2003). In contrast, animal models of genetic idiopathic epilepsy have only been used scarcely. This study was stimulated by the ability of LEV to suppress seizure activity in animal models mimicking absence epilepsy in man (Gower et al., 1995; Boon et al., 2002; Bouwman and van Rijn, 2004). Moreover, in a previous pilot study of chronic LEV administration to young GAERS, a preliminary indication of an anti-epileptogenic effect was found (Boon et al., 2002). Indeed, it was felt that investigating the effect of chronic LEV treatment in young GAERS could provide new insights and strategies for the treatment of epilepsy.

GAERS is an isomorphic and predictive model of absence epilepsy. The hallmark is the appearance of bilateral generalized ‘spike and wave discharges’ (SWDs) in the cortical EEG recordings during spontaneous absence seizures characterized by behavioral arrest. The first SWDs appear around postnatal day (PN) 30-PN40 and at the age of four months 100% of

the GAERS have SWDs, which persist throughout the lifespan of the animal (Danober et al., 1998).

In the present study, we have investigated the effect of LEV on the age-related development of SWDs in GAERS by chronic administration of LEV (PN23-PN60) starting before the age of occurrence of SWDs. The effect of early chronic LEV administration on the development of SWDs was evaluated in young GAERS during treatment (PN57-PN60), immediately after treatment termination (PN61-PN64) and two months after the last LEV injection (age four months), when brain maturation was achieved and SWDs recorded on cortical EEG were numerous.

2. Methods

2.1 Animals

Fifteen GAERS were used in this study. The animals were born and raised under environmentally controlled conditions (12 h light/dark cycles, lights on at 7 a.m., 20-22°C) with food and water *ad libitum* at the animal facility of the Ghent University Hospital. All animals were treated according to the guidelines approved by the European Ethics Committee (decree 86/609/CEE). The study protocol was approved by the Animal Experimental Ethical Committee of Ghent University Hospital.

2.2 Surgery

The pups (n= 15) were randomly assigned to the two treatment groups: LEV and control group. LEV (54 mg/kg, provided by UCB Pharma, Belgium) was dissolved in saline (0.9% NaCl) and injected intraperitoneally (i.p.) in a dose-volume of 2 ml/kg body weight, whereas control rats received an equivalent dose-volume of saline. From PN23 until PN60, LEV was administered once daily to eight GAERS, while the control GAERS (n= 7) received saline. All animals were weighed daily.

At age PN51, four epidural EEG stainless steel screw electrodes were bilaterally implanted over the fronto-parietal cortex of all 15 animals. The rats were anesthetized with ketamine/xylazine (80 mg/kg and 7.5 mg/kg respectively, i.p.) and additional ketamine (5 mg/kg, i.p.) was given when sensorial pain stimuli, by squeezing the foot pad, elicited motor reflexes. One epidural electrode was positioned on the left side of the skull at height of the frontal cortex (AP= +2 mm, ML= +1.2 mm, referred to bregma) and one was placed on the right sided parietal cortex (AP= -2 mm, ML= -1.2 mm, referred to bregma). The reference electrode was placed at lambda with the ground electrode on the left side of lambda (ML= +1.2 mm, referred to lambda). The EEG electrodes were fixed on the skull of the rat with

acrylic cement. Lidocaine gel (2%) was applied at the incision wound and the rats were placed under a heating lamp until they had recovered from anesthesia.

2.3 EEG recording

EEG was recorded by means of a H2O™ portable digital 32-channel EEG-recorder (Telefactor Corporation, U.S.A.). To avoid any interference with the circadian cycle, all recordings were performed during daytime (light period) starting at 10 a.m. in awake and freely moving animals (Rigoulot et al., 2003). Every 10 min a standardized sound stimulus was provided, to prevent the rats from falling asleep (Gower et al., 1995; Manning et al., 2004). During the recording sessions, the rats were continuously observed.

2.4 Effect of early chronic LEV administration on epileptiform events in young GAERS (PN57-PN64)

One week after surgery, the animals were placed in the EEG recording chambers and stayed there from PN56 (one day before the start of the EEG recordings) until PN64 with food and water *ad libitum*. During the last four days of the treatment (PN57-PN60), EEG was recorded for 3 h daily. In this period, LEV or saline was injected 1 h after the start of the EEG recording. From PN61 on, treatment was halted, animals (controls and LEV group) received no longer injections and EEG was recorded during four additional days (PN61-PN64), 3 h daily (Figure 1). The EEG was recorded on several days to have a more robust dataset on the effect of LEV in these young animals.

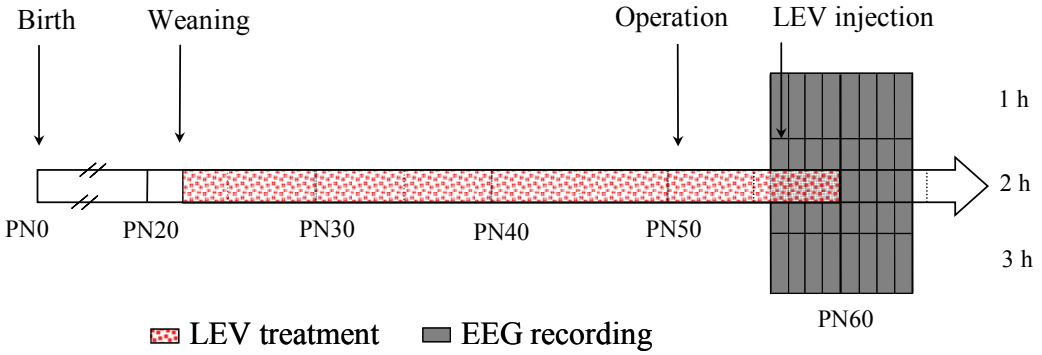

Figure 4: Study design. LEV (45 mg/kg, i.p.) was administered once daily at 11 a.m. from PN23 till PN60. During the last four days of the treatment (PN57-PN60) EEG was recorded for 3 h starting at 10 a.m.; the following four days (PN61-PN64), treatment was terminated and EEG was again recorded for 3 h a day.

Epileptiform events in young GAERS (PN57-PN64) were quantified by means of visual inspection of the EEG using TWIN™ off-line EEG analysis (Telefactor Corporation, U.S.A.) by an experienced electroencephalographer (SD). Young GAERS (under the age of 4

months) may not all have developed typical SWDs yet or have infrequent SWDs (Danober et al., 1998). For quantification purposes, epileptiform events were classified into two closely related types, because the morphology of SWDs in young GAERS was found to be less pronounced and more variable when compared with SWD patterns in adult GAERS (Figure 2): i) typical SWDs also present in adult GAERS (*amplitude*: > 3 times baseline; *peak frequency*: 7-12 Hz; *duration* > 0.5 s) and ii) SWD-like activity, which we have termed 'short irregular spike and wave discharges' (SISWDs), with clear spike and wave aspects characterized by lower amplitude (< 3 times baseline amplitude) or lower peak frequency (5-7 Hz) and shorter in duration (< 2 s). Only the numbers of different SWD types (SWDs and SISWDs) per hour were used as dependent values. The durations of SWDs and SISWDs were still very short in these young animals; therefore relevant differences in discharge duration between treated and control animals could not be detected.

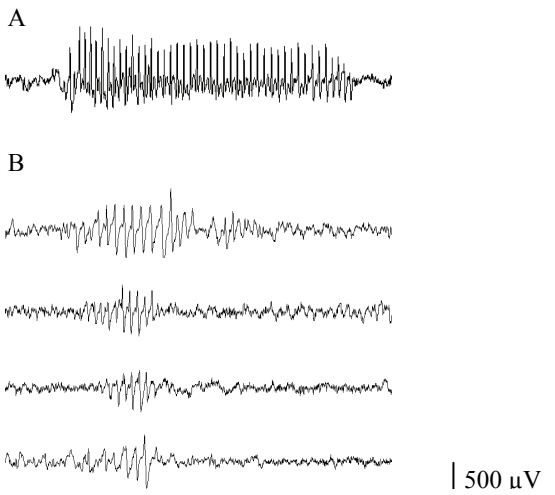

Figure 5: Different types of SWDs. A. Typical adult SWD (amplitude: > 3 times baseline amplitude; peak frequency: 7-12 Hz; duration > 0.5 s); B. SWD-like events i.e. SISWDs in young GAERS (PN57-PN64) consist of irregular short discharges, with clear spike and wave aspects characterized by lower amplitude (< 3 times baseline amplitude) or lower peak frequency (5-7 Hz).

2.5 Evaluation of SWDs in adult GAERS (age four months) after chronic LEV treatment during early life

About the age of four months (range PN120-PN124), EEG was recorded for one day (duration of recording between 6 and 8 h) in 15 animals. For the analysis of the SWDs on the EEG of the adult GAERS a previously described and validated detection software package was used (Van Hese et al., 2003). Once a list of marked time instances was obtained by the detection method, a number of post-processing steps were performed by the software package: detections lasting less than 0.5 s were discarded and two detections less than 1 s apart were taken together. SWDs detected by the software package were visually confirmed. In these animals, both the number per hour and mean duration of the SWDs during the recording hours were calculated as well as the cumulative duration of the SWDs per hour (summation of the duration of all SWDs per hour).

The software package specifically developed for the detection of SWDs was not appropriate for the scoring of the more irregular variant of the SWDs (SISWDs). Hence, the

EEG was visually inspected for SISWDs, fulfilling the conditions described above, and the number per hour was calculated.

2.6 Statistical analysis

In young GAERS, statistical analysis was performed on both pooled and unpooled data. Data could be *pooled* after exclusion of interactions (hour x treatment and day x treatment) by ANOVA with repeated measurements. The number of SWDs and SISWDs per 3 h was taken together; this was termed the number of *epileptiform events*. Statistical analysis was also performed on unpooled data, which consisted of the number of typical SWDs and SISWDs per hour at the different time points during a total recording time of 24 hours (3 h a day, during 8 consecutive days).

For the inter-group comparison between the LEV and control group the Mann-Whitney U-test was used. For the intra-group comparisons within the LEV and control group, a Friedman test was utilized. For the intra-group comparison between the mean number of epileptiform events on the two time points: during the last four days of treatment (PN57-PN60) and during the following four days after treatment had been terminated (PN61-PN64), a Wilcoxon signed rank test was used.

Through the further course of this paper (results and discussion), the results obtained in young GAERS will be presented and discussed according to the recording periods: a) the last four days of the treatment (PN57-PN60) and b) the following four days after treatment cessation (PN61-PN64).

In adult rats, the Mann-Whitney U-test was used to compare the number per hour, the mean duration and the cumulative duration/hour of the SWDs between the LEV and control group. For SISWDs, the number per hour was compared between the LEV and control group using the Mann-Whitney U-test.

All statistical tests were performed utilizing the statistical software package SPSS 12. Data are presented as the mean ± the standard error of the mean (SEM), and the significance level has been set at $\alpha = 0.05$.

3. Results

Chronic administration of LEV during 38 days in young GAERS (PN23-PN60) did not induce growth changes. Normal body weight in LEV treated GAERS (157 ± 8 g for males and 144 ± 10 g for females at PN60) was achieved in comparison with the control group (140 ± 15 g for males and 121 ± 6 g for females at PN60) treated with saline (NS, Student's t-test).

3.1 Effect of chronic LEV administration on epileptiform events in young GAERS (PN57-PN64)

LEV treated rats (n= 8) showed less SWDs and SISWDs than control rats (n= 7) during treatment and also during the four days after the discontinuation of the treatment. During this 8-day recording period, typical SWDs were observed in three control rats. Only one of these rats had frequent (12 ± 2 per hour) SWDs on the EEG, which were short in duration (1.6 ± 0.2 s). None of the LEV treated rats had typical SWDs; hence, there were too few SWDs to detect significant differences in SWDs between LEV and control group. SISWDs were observed in all rats. These SWD-like events were short lasting (< 1.5 s) in the LEV and in the control group.

a) Effect of chronic LEV administration on epileptiform events during the last four days of the treatment period (PN57-PN60)

During LEV treatment (PN57-PN60), the mean number of epileptiform events (pooled data) was 62% lower ($p < 0.05$, Mann-Whitney U-test) in the LEV group. The control group had a mean number of 86 ± 18 epileptiform events per 3 h, whereas this figure in the LEV group was 33 ± 11. Significant differences could also be found in the number of SISWDs (up to 81%) between LEV and control group on different time points during treatment (Figure 3). Also during the pre-injection hours (PN57-PN60), SISWDs were significantly lower in the LEV group (8 ± 2; 6 ± 2; 4 ± 2 and 7 ± 3) than in the control group (28 ± 8; 32 ± 6; 17 ± 6 and 20 ± 4) (Figure 3).

Pre- and post-injection hours differed significantly in treated animals on PN59 and PN60 (Friedman test). Surprisingly, the number of SISWDs in the post-injections hours was higher than in the pre-injection hour in the LEV treated group (Figure 3). However, a trend of a higher number of SISWDs in the post-injection hours was also present in the control group on PN59 ($p = 0.066$; Friedman test).

No differences were found between the four individual days during treatment (PN57-PN60) within LEV and control group (data not shown, Friedman test).

b) Effect of chronic LEV administration on epileptiform events after treatment cessation (PN61-PN64)

When treatment was discontinued (PN61-PN64), there was a borderline missed reduction of 53% in the mean number of epileptiform events (p= 0.064, pooled data, Mann-Whitney U-test) in the LEV group compared with the control group. The control group had a mean number of 73 ± 14 epileptiform events per 3 h, whereas this figure in the LEV group was 34 ± 8. However, significant differences could be found in SISWDs (up to 66%) between LEV and control group at different separate time points (unpooled data, Figure 3).

After treatment had been stopped, there were no differences between the recording hours within the LEV and the control group (Friedman test).

Within LEV and control group, there was a significant difference between the four days after treatment cessation (pooled data, Friedman test); this was manifested by an increase in the number of epileptiform events on the last two days of the recording period. In the LEV group these number of epileptiform events on PN61, 62, 63 and 64 were respectively 25 ± 8; 23 ± 7; 40 ± 10 and 48 ± 9. In the control group these figures were 52 ± 14; 57 ± 10; 94 ± 19 and 88 ± 19.

The Wilcoxon signed rank test did not reveal differences between the mean number of epileptiform events during treatment period and after treatment cessation in both groups (86 ± 13 versus 73 ± 14 for the control group and 33 ± 11 versus 34 ± 6 for the LEV group).

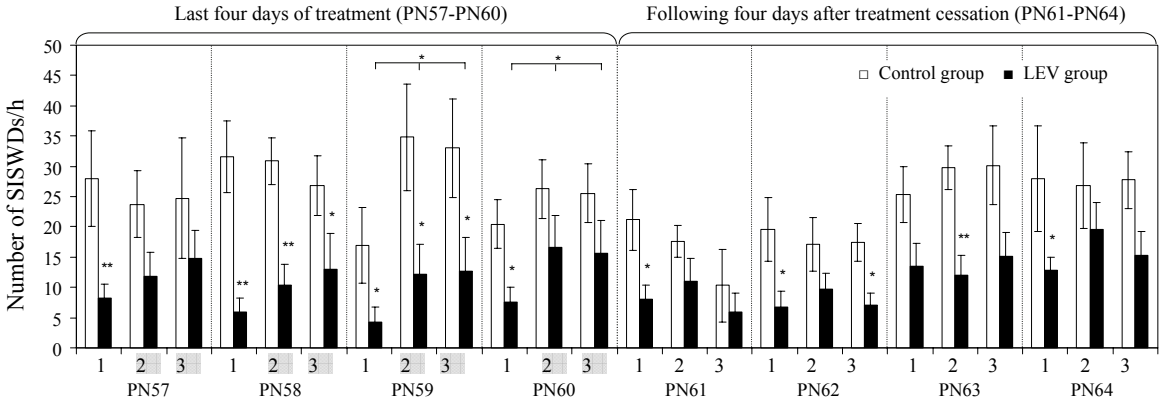

Figure 6: Effect of chronic LEV administration (PN23-PN60) on SISWDs in young GAERS (PN57-PN64). Number of SISWDs in the LEV group treated with LEV (solid bars, n= 8) and in the control group treated with saline (open bars, n= 7) during treatment (PN57-PN60) and after treatment discontinuation (PN61-PN64). Data are expressed as mean ± SEM; SISWDs= short irregular spike and wave discharges; PN= post natal day; 1 h= pre-injection hour or first recording hour; 2 h= first post-injection hour or second recording hour; 3 h= second post-injection hour or third recording hour. Differences between control and LEV group are assessed using the Mann-Whitney U-test and differences between the three different hours within the groups is assessed using the Friedman test; * indicates $p< 0.05$, ** indicates $p< 0.01$.

3.2 Evaluation of SWDs in adult GAERS (age four months) after chronic LEV treatment during early life

At the age of four month all animals had typical SWDs. EEG recordings showed no significant difference between the LEV and control group in number, mean duration and cumulative duration of the SWDs (Mann-Whitney U-test). These numbers were only slightly lower in the LEV group (Figure 4).

Also in SISWDs no differences could be found between both groups (Figure 4). However, SISWDs were decreased substantially in favor of typical SWDs in comparison with young GAERS (PN57-PN64), which had more SISWDs than SWDs.

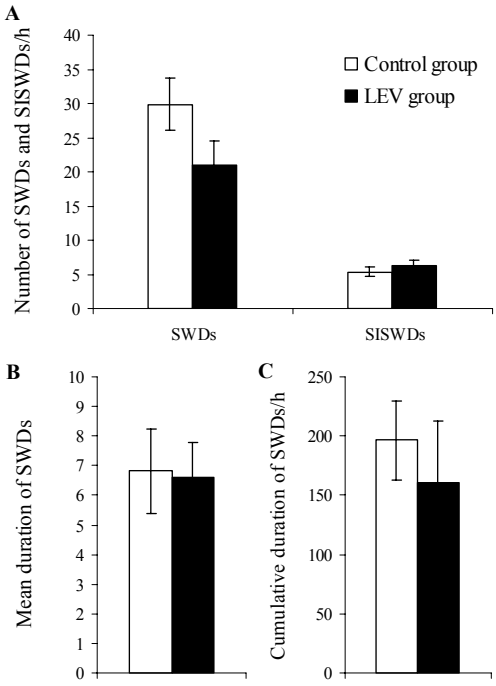

Figure 7: Evaluation of epileptiform events in adult GAERS (age 4 months) after chronic LEV treatment during early life. A. Number of SWDs and SISWDs per hour, B. mean duration of SWDs (s) and C. cumulative duration of SWDs per hour (s/h) in the LEV group (solid bars, n= 8) and in the control group (open bars, n= 7). Data are expressed as mean ± SEM.

4. Discussion

A new approach to drug screening, including the process of epileptogenesis, may identify new classes of drugs (Kupferberg, 2001; Klitgaard and Pitkanen, 2003). LEV is a novel AED with potential anti-epileptogenic properties, which was discovered using non-conventional drug screening. It suppresses kindling development at doses devoid of adverse effects with persistent reduction in afterdischarge duration after termination of the treatment (Loscher et al., 1998). In the SER-model, LEV was administered before the appearance of spontaneous seizures and was terminated at the expected age for seizure expression, which resulted in a lower seizure number in pre-treated animals (Sasa et al., 2003). In the present study, we found that chronic LEV administration induced a decrease in epileptiform events (SWDs and SISWDs) in young GAERS (PN57-PN60), which persisted immediately following cessation of treatment (PN61-PN64), but not until adulthood.

In GAERS, SWDs strictly correlate with the occurrence of clinical absences (Danober et al., 1998), which was also observed in this study. However, behavioral absence seizures can be subtle as they mostly appear during quiet wakefulness (Danober et al., 1998). Therefore, EEG recordings were used to quantify the appearance of SWDs. In our study, we

found that typical SWDs are rare in young GAERS. Only three out of seven young control GAERS showed typical SWDs on the EEG and none of the LEV treated animals (n= 8). SISWDs, however, were the most prevalent type of discharge on the EEG during quiet wakefulness. Hence, these more irregular discharges were taken into account to quantify the effect of LEV on epileptiform events. While it is difficult to correlate SISWDs with behavioral arrest because of their short duration (< 1.5 s), it can be assumed that these discharges are closely related to SWDs and reflect premature SWDs given their morphological resemblance with typical SWDs and their strong decrease in favor of typical SWDs as the rats grow older.

In this study, epileptiform events (SWDs + SISWDs) were significantly suppressed (difference of 62%) during chronic LEV treatment in young GAERS (PN57-PN60) in comparison with control animals. This is in line with a previous acute study of Bouwman and van Rijn (2004), which found a similar difference in SWDs between control and LEV treated adult rats in the WAG/RIJ model of absence epilepsy.

The LEV treated animals displayed less SISWDs (up to 81%) than the control group even in the pre-injection hours during PN57-PN60. This could suggest that there was a slowing down of the epileptogenic processes by early chronic treatment (PN23-PN60) with LEV. Yet, the lower number of SISWDs in the pre-injection hours might also be caused by retention of LEV in the brain from previous injections, although LEV was already eliminated from the blood plasma.

Interestingly, there was no acute decrease in SISWDs after injection of LEV (PN57-PN60) and even the opposite could be observed. This is in contrast with previous acute experiments in adult GAERS, in which a clear decrease in SWDs could be observed after a single LEV injection (Gower et al., 1995; Boon et al., 2002; Bouwman and van Rijn, 2004). These studies, which were performed in adult GAERS, report the effect of a first and single LEV injection, while the effect of chronic LEV administration was not investigated. Moreover, several studies dealing with the chronic administration of LEV in animal models of temporal lobe epilepsy report increased functional tolerance towards LEV and some loss of efficacy after prolonged administration (Loscher et al., 1998; Loscher and Honack, 2000; Glien et al., 2002). This could be a possible reason for the lack of an acute decrease in SISWDs in rats that were already pretreated.

The acute increase in SISWDs on PN59 and PN60 after LEV administration could represent a flaring up which stabilized again towards the next day. A trend for this phenomenon was also observed in the control group on PN59 (p= 0.066). There was no difference between recording hours during the last four days, when the rats were no longer injected with saline or LEV (PN61-PN60). Hence, stress after injection could be a responsible

factor for this increase in SISWDs, although the animals were accustomed to the handling procedures.

When treatment was stopped, there was no sudden increase in epileptiform events (SWDs and SISWDs). This is in line with previously performed rat and mice experiments, where chronic treatment with LEV did not lead to withdrawal hyperexcitability (Klitgaard et al., 1998; Loscher and Honack, 2000). During different time points, epileptiform events were still significantly lower (up to 66%) in young LEV treated GAERS. This difference in number of absence seizures between controls and LEV treated rats is comparable to the study in the SER model (Sasa et al., 2003). In the SER-study, absence seizures were still decreased two weeks after treatment cessation during the 30 min recording period. However, the next week there was no longer a significant difference between the control and LEV pretreated group. In the study of Glien et al. (2002), spontaneous partial seizures in the pilocarpine model of temporal lobe epilepsy were also still decreased one week after termination of chronic LEV administration (two weeks) in certain rats, this was described as a carryover effect.

In both LEV and control group, epileptiform events started to increase on the last days of recording, which can be due to the progressive nature of absence epilepsy in young GAERS. Still, significant lower SISWDs (up to 66%) in the LEV group could be detected on these last days of EEG recording (PN63 and PN64) in comparison with the control group.

Early chronic treatment with LEV in young GAERS might have caused an initial slowing down of the epileptogenic processes, which had persisting effects after treatment termination. The major metabolite of LEV is pharmacologically inactive, and other metabolites are produced at levels below those that are relevant for pharmacological activities (Loscher et al., 1998). LEV does not interfere with enzyme systems (Patsalos, 2000; Benedetti, 2000) and is washed out rapidly (half-live ~2-3 h) after stopping the treatment (Loscher et al., 1998). As we used a relatively high dose to inject and LEV is already effective in low doses in GAERS (Gower et al., 1995), we expect that relevant therapeutic concentrations are present in the blood plasma for 8 h. Hence, the prolonged decrease in epileptiform events on PN61-PN64 is probably not due to a prolonged acute effect caused by accumulated LEV or other metabolites.

Early chronic administration of LEV had no effect on the later expression of SWDs at the age of four months. SISWDs have diminished considerably in both groups; this supports its role as premature SWDs. Most likely, after discontinuation of the treatment, epileptogenesis could further progress. To our knowledge, there is only one other study where an AED i.e. remacemide was chronically administered in young GAERS (PN7-PN25) to establish its effect on SWD development (Nehlig and Boehrer, 2003). This study failed to demonstrate an effect of early remacemide treatment on the expression of SWDs in adult GAERS (Nehlig and Boehrer, 2003). In the present study, adult GAERS displayed fewer

seizures and shorter mean SWD durations compared to previously published experiments in GAERS, which were monitored only for a short period of time (Danober et al., 1998). However, these values were similar to a previous chronic experiment performed by our group (Dedeurwaerdere et al., 2004). This phenomenon of a decrease in seizures during longer recording periods is correlated with the decrease of vigilance resulting in more sleep episodes and less and shorter seizures (Dufour et al., 2001; Rigoulot et al., 2003).

Until now, it is not described in what manner LEV exerts its effect on absence seizures in GAERS. LEV has no effect on the T-type voltage-activated Ca^{2+} current (Zona et al., 2001), a common target for absence epilepsy drugs. A possible effect of LEV that might contribute to its action against SWDs in GAERS could be by progressively decreased firing of the substantia nigra pars reticulata (SNR) neurons (Loscher et al., 1996). The SNR is not part of the epileptic circuit underlying absence seizures, but it may belong to a system participating in a remote control over seizures (Danober et al., 1998). Indeed, high frequency stimulation of the SNR has been shown to interrupt SWDs in GAERS (Feddersen et al., 2004) and intracerebral microinjections of GABA mimetics in the SNR suppress absence seizures (Danober et al., 1998). A part of LEV's ability to suppress seizures may origin in its effect on neuronal firing in the SNR, but the anatomical network and molecular mechanisms by which this is mediated remains to be demonstrated (Klitgaard et al., 1998). By this way LEV could suppress SWDs in GAERS, but it is not known if this might interfere with epileptogenesis. In GAERS pups, metabolic increases in SNR compared to non-epileptic control rats were observed, which was suggested to reflect a mechanism of prevention of epileptogenesis in GAERS (Nehlig et al., 1998).

4. Conclusion

In this study, chronic LEV administration induced a decrease in epileptiform events in young GAERS (PN57-PN60), although this was not obtained by a decrease immediately after injection. This effect persisted partly after cessation of treatment (PN61-PN64), which might reflect a slowing of epileptogenic process due to previous chronic treatment with LEV. However, when LEV and saline treated animals grew up, they all developed a similar expression of spontaneous absence seizures.

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Chapter 4: Efficacy of vagus nerve stimulation

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Acute vagus nerve stimulation does not suppress spike and wave discharges in "Genetic Absence Epilepsy Rats from Strasbourg"

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We evaluated the efficacy of vagus nerve stimulation (VNS) in genetic absence epilepsy rats from Strasbourg (GAERS), a validated model for absence epilepsy. In the first experiment, we investigated whether VNS applied at seizure onset can interrupt spike and wave discharges (SWDs). In the second experiment, we investigated whether SWDs are suppressed or shortened in duration when VNS is applied several hours per day. Both control and VNS groups underwent EEG and VNS electrode implantation. For the first experiment, a randomized crossover design was used. Stimuli (amplitude: 3 V; frequency: 30 Hz; pulse duration: 500 μs) were given when an SWD occurred on the EEG. The experiment was repeated the next day. In the second experiment, treated animals were stimulated (amplitude: 1.5 mA; frequency: 30 Hz; pulse duration: 500 μs; on/off time cycle: 30 s/ 5 min) for three hours per day, during five consecutive days. In the first experiment, the duration of the SWDs was increased on day 1, ($p < 0.05$). There was no difference in SWD duration on day 2. In the second experiment, no significant differences could be found in number, duration and EEG frequency of SWDs. VNS applied at the onset of an SWD can prolong the duration of SWDs in GAERS. As a five-day stimulation protocol had no effect, long-term VNS might be necessary to affect SWDs.

Key words: vagus nerve stimulation, absence epilepsy, GAERS, EEG

1. Introduction

Vagus nerve stimulation (VNS) is an efficacious broad-spectrum add-on treatment for patients with medically or surgically refractory epilepsy (Ben-Menachem, 2002). The left vagus nerve is stimulated intermittently by means of an implanted pulse generator and reduces the frequency and severity of epileptic seizures. Controlled randomized trials showed a 50% decrease in overall seizure frequency in approximately 30% of the patients (Salinsky, 2003).

The precise mechanism of action of VNS is not known. Mechanistic VNS research generally presumes that VNS exerts its effect by inducing action potentials in the afferent fibres of the left vagus nerve (Zagon et al., 1999; Henry, 2002). Through diffuse projections of the vagus nerve in the cerebral hemispheres, VNS can have a broad effect on neuronal excitability (Rutecki, 1990).

VNS is being used to treat generalized and partial epilepsy, but responder groups are not clearly identified. The efficacy profile of VNS in non-convulsive generalized epilepsy, such as absence epilepsy is limited. Fundamental research of VNS in convulsive generalized and partial animal models preceded and supports the use of VNS in epilepsy patients (Lockard et al., 1990; Woodbury and Woodbury, 1990; Woodbury and Woodbury, 1991; Zabara, 1992; McLachlan, 1993; Takaya et al., 1996; Fernandez-Guardiola et al., 1999). Such fundamental research of VNS in absence epilepsy is presently lacking.

Absence seizures are characterized by paroxysmal unresponsiveness to environmental stimuli and cessation of ongoing activity. Absence seizures are associated with the appearance of bilaterally synchronous 3 Hz spike and wave discharges (SWDs) on the EEG. They occur mainly during quiet wakefulness, inattention and in the transitions between sleep and waking (Guey et al., 1969). Human absence epilepsy has a genetic predisposition, without evidence of any structural lesion as its substrate (Niedermeyer, 1996). A thalamocortical dysfunction is assumed to play a major role in the underlying pathophysiology (Danober et al., 1998).

Research on absence epilepsy is greatly facilitated by the use of animal models. Genetic absence epilepsy rats from Strasbourg (GAERS) constitute an experimental animal model and fulfill the requirements for an isomorphic and predictive model of absence epilepsy (Marescaux, 1992). The absence seizures in GAERS are accompanied by typical SWDs (7-12 Hz) on the EEG. The absences are characterized by behavioral arrest and are sometimes accompanied by rhythmic twitching of the vibrissae. Unexpected environmental stimuli can interrupt the seizures. Absence seizures in GAERS differ from human absences in SWD frequency (7-9 Hz versus 3 Hz) and the occurrence during lifetime (adulthood versus childhood with tendency to disappear with adulthood). But both GAERS and human absence seizures are believed to arise from the same pathophysiological background.

In this short-term study, it was our intention to investigate the efficacy of VNS in GAERS. In the first experiment, we investigated whether SWDs can be interrupted by acutely applying VNS at a SWD onset. In the second experiment we investigated whether SWDs are suppressed or shortened in duration when VNS is applied during several hours/day during five consecutive days.

2. Methods

2.1 Animals

Twenty-one GAERS (male and female), weighing 200-300 g and aged four to six months, were used in this study. The animals were born and raised under environmentally controlled conditions (12 h light/dark cycles, 20-22°C) in the animal facility of the Ghent University Hospital with food and water ad libitum. All animals were treated according to guidelines approved by the European Ethics Committee (decree 86/609/EEC). The study protocol was approved by the Animal Experimental Ethical Committee of Ghent University Hospital (ECP 01/26).

2.2 Surgical and technical procedures

The animals were implanted with a cuff-electrode around the left vagal nerve and five epidural EEG electrodes. The GAERS were anasthetized with pentobarbital (40 mg/kg, i.p.). For implantation of the stimulation cuff-electrode an incision was made over the left anterior cervical region to allow free dissection of the left vagus nerve over 1 cm. The ends of the cuff-electrode were tunnelled under the skin over the back of the neck to the head, where they were fixated with acrylic cement.

Stimulation was performed by connecting the cuff-electrode to an external Tektronix stimulator (Tektronix, Inc., USA) (experiment 1) or to an external NeuroCybernetic Prosthesis device (NCP, model 100; Cyberonics Inc., USA) (experiment 2). The impedance of the electrode-to-vagus nerve interface was measured at the beginning and end of each experiment; impedance values were the same before and after the experiments for all animals and were always between 1 and 3 kΩ. In the first experiment, output voltage was set at 3 V (~1.5 ± 0.75 mA). Behaviourally, animals did not react to the stimulus given. In the second experiment, a current source (an external NeuroCybernetic Prosthesis device) was used, to assure that a constant current (1.5 mA) was provided. When a stimulus of higher intensity was given, animals behaviourally reacted by turning away of the head and sometimes by scratching toward the neck. A short test stimulus (duration: 1s; amplitude: 5 V or 2.5 mA, frequency: 30 Hz and pulse duration: 500 μs) was delivered after each experiment to confirm that true stimulation had been delivered.

Epidural EEG stainless steel screw electrodes were bilaterally implanted over the fronto-parietal cortex. Two epidural electrodes were positioned left and right on the skull opposite to frontal cortex (AP= +2 mm, ML= ±1.2 mm, DV= 0 mm, referred to bregma) and two were placed left and right on the parietal cortex (AP= -2 mm, ML= ±1.2 mm, DV= 0 mm, referred to bregma). The reference electrode was placed at lambda. The EEG electrodes were fixed together with the cuff-electrode leads on the skull of the rat with acrylic cement. For the

EEG recording a H2O™ portable digital 32-channel EEG recorder (Telefactor Corporation, USA) was used. The data were quantified by visual inspection of the EEG using TWIN™ off-line EEG-analysis (Telefactor Corporation, USA). In GAERS, during the awake state EEG amplitude is lower then 300 µV. During sleep, there is a high voltage synchronized activity, often accompanied with sleep spindles, but the appearance of SWDs on the EEG of sleeping GAERS is extremely rare (Pinault et al., 2001). Therefore, the following criteria were used for the detection of SWDs: duration: ≥ 1 s; amplitude: > 300 µV; EEG frequency: 7-12 Hz.

The animals were allowed to recover for three to seven days before the experiments were performed. They were placed in the recording chambers one day before the experiments began and stayed there during the whole experimental period. The experiments were carried out during morning and early afternoon hours (light period). During the experiments, the animals were not receiving anesthetics and were freely moving.

2.3 Experimental design

2.3.1 Experiment 1: acute VNS at the onset of a SWD

Stimulation parameters were set at 3 V, 30 Hz frequency and 500 µs pulses. The EEG was visually inspected and VNS was manually initiated every time a SWD occurred on the EEG and was stopped when the SWD ended. An automated detection system for SWDs in GAERS triggering VNS online when a SWD appeared on the EEG, was not available. Stimuli were given with an external Tektronix stimulator (Tektronix, Inc., USA).

A randomized crossover design was used. Thirteen animals were implanted as described above. During two consecutive days, 2 h of EEG were recorded. During one hour, VNS was applied (VNS condition) and during the other hour, the animals were not stimulated (baseline condition). In the VNS group, baseline and VNS conditions were randomly assigned. The stimulation protocol of the stimulated animals (n= 8) is described in figure 1. To provide baseline values and to allow inter-group comparison a control group (n= 5) was included.

Following the experiments, the duration of the SWDs (start and end of the SWD), was manually marked on the EEG recordings. The average latency to initiate VNS when a SWD appeared on the screen was 2.5 ± 1.0 s. Consequently, all SWDs with duration shorter than 2.5 s were left out of the analysis during baseline and VNS conditions, in both the control and stimulated group.

For the statistical analysis of the intra-group comparison, we used the non parametric Wilcoxon signed rank test for paired samples. In the VNS group, paired comparisons were made for baseline conditions and VNS conditions in the same rat, on day 1 and day 2. Inter-group comparison between the baseline values of the control group and the VNS condition values of the VNS group was performed by using a Mann-Whitney U test for day 1 and day 2.

2.3.2 Experiment 2: continuous application of VNS during ongoing SWD recording

For the purpose of this protocol, VNS was delivered by means of a programmable stimulating device (NeuroCybernetic Prosthesis, model 100; Cyberonics Inc., USA) using the following parameters: amplitude: 1.5 mA; frequency: 30 Hz; pulse duration: 500 μs; on/off cycle: 30 s/ 5 min.

On day 1, baseline EEG was recorded during four hours. For a period of five consecutive days, six rats were stimulated for 3 h/day. On day 2, 3 and 6, EEG was recorded during 4 h starting 1 h before stimulation (baseline). Two control GAERS underwent implantation of the recording and stimulation electrodes but were not stimulated, EEG was recorded during 4 h on day 1, 2, 3 and 6.

On day 1, 2, 3 and 6, the number, the mean duration and EEG frequency of SWDs were compared between the first hour (hour 1, baseline) and the last hour of stimulation (hour 4) in the control and the VNS group. For this purpose, a Wilcoxon signed rank test was used for paired samples. The Fisher's exact test was used to compare the last hour of stimulation (hour 4) of day 1 with the last hour of stimulation (hour 4) of day 2, day 3 and day 6 in the control and the VNS group.

3. Results

3.1 Experiment 1: acute application of VNS at the onset of a SWD (Figure 1)

On the first day of the experiment, the mean duration of the SWDs increased significantly ($p < 0.05$) in the stimulated hour versus the non-stimulated hour of the VNS group. The mean duration of SWDs without VNS was 13.3 s (SD= 4.1) and 19.4 s (SD= 4.7) with VNS in the stimulated group.

When the experiment was repeated, there was no change in SWD duration on the second day of the study (day 2). When VNS conditions of the VNS group were compared with the baseline values of the control group, there were no significant differences on day 1 and day 2.

3.2 Experiment 2: continuous application of VNS during ongoing SWD recording (Figure 2)

When baseline conditions (hour 1 of day 2, 3 and 6 and hour 4 of day 1) were compared to VNS conditions (hour 4 of day 2, 3 and 6) in the stimulated GAERS (n= 6) and in the control group (n= 2), no statistically significant differences could be found in number, duration and EEG frequency (Figure 2) of the SWDs.

On day 2, in both controls and VNS group, the average number of SWDs in all recording hours was lower than on the other days.

	DAY 1		DAY 2	
	Group A (n=4)	**Group B (n=4)**	**Group A (n=4)**	**Group B (n=4)**
1st hour	**VNS conditions**	Baseline conditions	Baseline conditions	**VNS conditions**
2nd hour	Baseline conditions	**VNS conditions**	**VNS conditions**	Baseline conditions

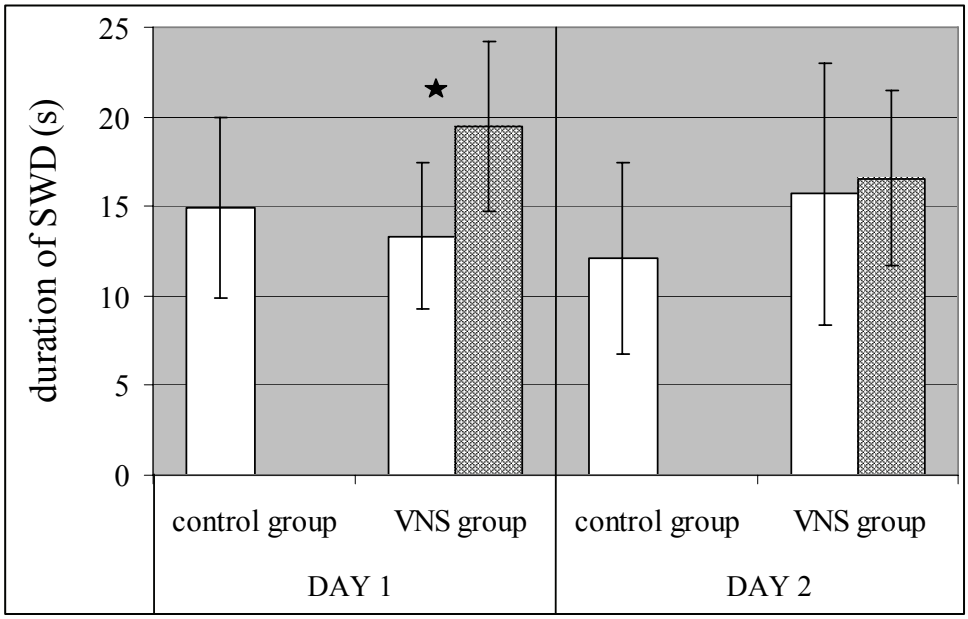

Figure 8: Acute effect of VNS on SWD duration in GAERS, experiment 1. Upper panel: study design (cross over); lower panel: X-axis: treatment group and Y-axis: mean duration of SWD per hour (s). Data are expressed as mean ± SD, * indicates significance, which is set at $p < 0.05$.

4. Discussion

The precise mechanism of action by which VNS exerts its anti-epileptic effect has not been elucidated. A better insight in the mechanism of action may help to identify specific epilepsy syndromes or types of epilepsy that respond well to VNS, a major issue that has not been resolved. Absence seizures in humans are usually a benign form of epilepsy (Wolf and Inoue, 1984). Despite the good pharmacological response in this type of epilepsy, investigating the efficacy of VNS in GAERS aims at identifying clues on the mechanism of action of VNS.

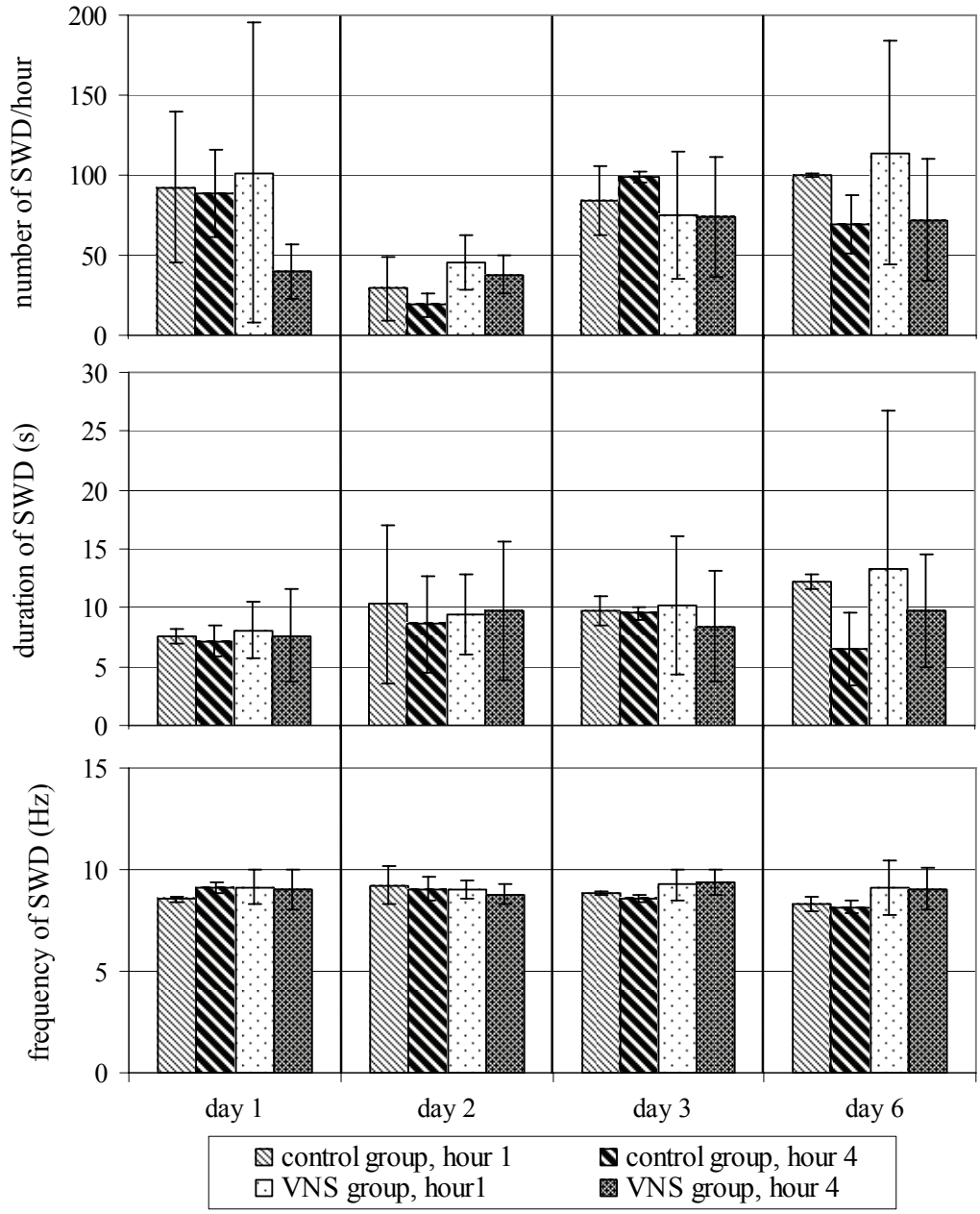

Figure 9: Effect of VNS on the number, duration and EEG frequency of SWD/hour in GAERS, experiment 2. X-axis: day of the experimentation period; Y_1-axis: mean number of SWD per hour; Y_2-axis: mean duration of SWD (s) and Y_3-axis: mean frequency of SWD (Hz). Data are presented as mean ± SD.

Initial animal studies with stimulation of vagal afferents showed promising results in reducing both ictal and interictal EEG abnormalities (Lockard et al., 1990; Woodbury and Woodbury, 1990; Woodbury and Woodbury, 1991; Zabara, 1992; McLachan, 1993; Takaya et al., 1996; Fernandez-Guardiola et al., 1999). These findings laid the foundation for the further development of VNS as a treatment for human epilepsy. So far, VNS has not been investigated in GAERS. As in this model the SWDs correlate strictly with the occurrence of clinical absences, we can state that this study investigated the effect of VNS both on ictal

EEG and seizures. In the present study we were not able to suppress or shorten the typical EEG pattern associated with absence seizures in these animals when VNS was applied acutely or in a prolonged pattern over several days. On day 2 of experiment 2, there was a reduction in number of SWDs both in the VNS group and in the control group. It is unlikely that this reduction is due to the treatment. It could be related to external factors, as the occurrence of absence seizures in GAERS is sensible for changes to environmental conditions.

VNS decreases interictal epileptiform spikes and spike and wave activity on the EEG over time, but may induce an initial synchronization of this epileptiform activity (Koo, 2001). This previous finding in man could correlate with our finding of an initial prolongation of the SWDs in GAERS during acute VNS in the first experiment. This prolongation was only seen on the first day of experiment 1. Precautions were taken to assure true stimulation during the whole experiment. This included the measurement of the impedance and a test stimulus at the end of the day. In a previous study, utilizing the same stimulation protocol, a decrease in body weight was observed after two weeks of VNS (Dedeurwaerdere et al., 2003). There remains however a need for a more direct and accurate measuring of the efficacy of the stimulation, both in animals and humans. Adapting the electrode to include measuring capability of action potentials of the different nerve fibres induced by VNS, could provide an answer to this question.

VNS efficacy in animals has mainly been assessed using acute models. In contrast, GAERS are a genetic model in which it takes four months before full epileptogenesis is established and can be considered as a chronic model with spontaneous seizures. Anti-epileptic and even anti-epileptogenic effects of VNS were found in a chronic kindling model (Fernandez-Guardiola, 1999). But when VNS was tested for its efficacy in dogs with refractory epilepsy, seizure reduction was found only after nine weeks of stimulation (Munana et al., 2002). These animals studies, investigated VNS efficacy in convulsive seizures. Absence seizures, which are non-convulsive, use neuronal mechanisms that are different from convulsive events (Eskazan et al., 2002). The effect of VNS on convulsive seizures may be different from the effect on non-convulsive seizures. This differential effect has also been shown for certain anti-epileptic drugs (Marescaux et al., 1992).

There are some reports of the use of VNS in human absence epilepsy (Labar et al., 1998; Frost et al., 2001; Helmers et al., 2001; Labar et al., 2001; Farrag et al., 2002). Ben-Menachem et al. (1999) described a positive effect of VNS in a small number of patients such as primary generalized epilepsy with absence seizures and generalized tonic clonic seizures. As these patients were treated with VNS, the absence seizures were most likely medically refractory. Typical absences and more refractory absence seizures may be distinct clinical entities. It is unknown to what extent GAERS are also a valuable model for refractory absence seizures. Apart from these reports in the literature, suggesting that generalized epilepsy

responds equally well to VNS as partial epilepsy, it appears that in several patients with absence epilepsy a significant decrease in seizure frequency was found only after six to twelve months of treatment with VNS (Parain et al., 2003).

Some imaging studies show a differential effect of acute and chronic VNS on cerebral blood flow (Henry, 2000; Vonck et al., 2003). It could be that acute and chronic VNS have a different mechanism of action. Whereas acute VNS likely reflects a purely anti-convulsive effect, the major therapeutic effect of VNS has been assigned to chronic VNS, which is probably related to an anti-epileptic effect (Boon et al., 2002).

Finally, the role of different stimulation parameters needs to be further investigated. It may be that different stimulation parameters are more efficacious in different types of epilepsy. Our experimental setup used stimulation parameters comparable to the ones used in humans.

5. Conclusion

Acute application of VNS at the onset of SWDs and continuous application of VNS during ongoing SWD recording in GAERS failed to show seizure suppressing effects. VNS applied at the onset of SWDs can prolong the duration of SWDs in GAERS. It could be of great value to stimulate GAERS over an extended period to observe chronic effects. Epileptogenesis in GAERS is a process that takes months so it is not unlikely that a longer stimulation period is required to make a difference and to affect an established pathology. This reasoning is supported by several studies in humans that describe a cumulative effect of VNS resulting in increased efficacy over a longer period of time. Further research using different stimulation parameters and/or a longer stimulation period is necessary to elucidate the real nature of the effect of VNS on SWDs in GAERS.

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The Acute and Chronic Effect of Vagus Nerve Stimulation in Genetic Absence Epilepsy Rats from Strasbourg (GAERS)

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PURPOSE: The aim of this study is to evaluate the efficacy of *acute* and *chronic* vagus nerve stimulation (VNS) in genetic absence epilepsy rats from Strasbourg (GAERS). This is a validated model for absence epilepsy, characterized by frequent spontaneous absences concomitant with spike and wave discharges (SWDs) on the EEG. Although absences are a benign form of seizures, it is conceptually important to investigate the efficacy of VNS in a controlled study by using this chronic epilepsy model.
METHODS: Both control and stimulated GAERS were implanted with five epidural EEG electrodes and a stimulation electrode around the left vagus nerve.
In the first experiment, VNS was given when SWDs occurred on the EEG; this was repeated the next day. A randomized cross-over design (n= 8) was used.
In the chronic experiment, GAERS underwent EEG monitoring during a first baseline week. During the second week, the treated group (n= 18) received VNS, controls (n= 13) on the other hand only underwent EEG recordings.
RESULTS: On day 1 of the acute VNS experiment, the mean duration of the SWDs when VNS was applied was higher than in baseline conditions ($p < 0.05$). However, on day 2, there was no difference in mean duration of the SWDs.
In the chronic VNS experiment no statistically significant differences were found between control and stimulated GAERS.
CONCLUSION: Acute VNS applied shortly after the onset of SWDs prolonged the mean duration of SWDs in GAERS at least during the first day of VNS. Chronic stimulation hardly affected SWDs in GAERS.

Key words: vagus nerve stimulation (VNS), GAERS, absence epilepsy

1. Introduction

Vagus nerve stimulation (VNS) is an adjunctive treatment for refractory epilepsy. It is used in generalized and partial epilepsy (Ben-Menachem, 2002), although responder groups have not been clearly identified. Knowledge about the efficacy profile of VNS in non-convulsive generalized epilepsy, i.e. absence epilepsy, is limited because patients with this type of epilepsy generally have a good pharmacological response. Extensive therapeutic use of VNS in absence epilepsy is therefore unlikely, but information about the potential efficacy of VNS in this type of epilepsy could help to clarify the general principles that underlie VNS.

GAERS are a validated genetic model for absence epilepsy characterized by typical spike and wave discharges (SWDs) on the EEG during absence seizures (Danober et al., 1998).

This work evaluates the efficacy of *acute* and *chronic* VNS in this model of primary generalized epilepsy. We investigated if acute VNS can abort SWDs and if chronic stimulation suppresses absence seizures.

2. Methods

Thirty-nine GAERS (male and female), weighing 200-300 g and between four to six months of age, were used in this study. All animals were treated according to guidelines approved by the European Ethics Committee (decree 86/609/EEC), confirmed by an approval of the local ethical committee for animal studies.

The animals were implanted with a cuff-electrode around the left vagal nerve and with five epidural EEG electrodes as described previously (Dedeurwaerdere et al., 2004). This cuff-electrode has been successfully used in suppressing pentylenetetrazole (PTZ) evoked seizures during experiments of Takaya et al. (1996).

After one week of recovery, the animals were placed in a recording cage one day before EEG recordings began and stayed there for the whole experimental period. Stimulation parameters used in this study were based upon previous experiments (Woodbury and Woodbury, 1990; Woodbury and Woodbury, 1991; McLachlan et al., 1993; Takaya et al., 1996). The impedance of the electrode-to-vagus nerve interface was measured at the beginning and end of each stimulation session. A short test stimulus of higher intensity (2.5 mA, 500 μs pulses at 30 Hz for 1 s), which provoked a behavioral reaction of the rat (turning away of the head or flattening of the ears and sometimes scratching toward the neck), was used to check proper functioning of the stimulation configuration. Strong and unexpected sensory stimulations immediately interrupt SWDs in GAERS (Danober et al., 1998). This was also the case for the high intensity test stimulus and we therefore selected a subthreshold stimulation of 1.5 mA in this study.

2.1 Experiment 1: acute VNS

Because the response time of the stimulator normally used in VNS (see below) was too long (> 1 s) for the acute interference experiments, stimuli (500 μs pulses at 30 Hz) had to be applied by a standard voltage source (Tektronix, Inc., USA). Cuff-impedance was measured before and after each experiment (between 1 and 3 kΩ) and stimulation voltage was adjusted to deliver a current of 1.5 mA. EEG was visually inspected by an experienced electroencephalographer (SD) and VNS was manually switched on every time a SWD

occurred; it was stopped once the SWD had ended. A randomized crossover design (n= 8) was used: two hours of EEG were recorded on two consecutive days. During one hour, VNS was applied (VNS condition) and during the other hour, the animals were not stimulated (baseline condition).

Following the experiments, the start and end of the SWDs and VNS were marked on the EEG recordings. The average latency between the start of the SWD and the activation of VNS was 2.5 ± 0.4 s. Consequently, all SWDs with duration shorter than 2.5 s were left out of the analysis during baseline and VNS conditions.

Averaged data are presented as mean ± standard error of the mean (SEM). For the statistical analysis, we used the non-parametric Wilcoxon signed rank test for paired samples to compare mean duration in baseline conditions and VNS conditions in the same rat, on day 1 and on day 2. $P< 0.05$ is assumed to indicate a significant difference.

2.2 Experiment 2: chronic VNS

In the chronic VNS experiment, stimuli were delivered by a constant current stimulator, normally used in VNS (NeuroCybernetic Prosthesis system, model 100, Cyberonics Inc., USA). Following settings were used: amplitude: 1.5 mA; frequency: 30 Hz; pulse duration: 500 μs; on/off cycle: 60 s/ 12 s. During a first week (baseline period), animals (n= 31) were not stimulated. During a second week, 18 GAERS were stimulated 24/24 h and control GAERS (n= 13) were only recorded. EEG was recorded for 3 h on day 7 and again on day 14. The detection of the SWDs on the EEG was performed using a custom designed software package in Labview (Van Hese et al., 2003).

The number of SWDs per hour, their mean duration, cumulative duration per hour and peak frequency is compared. Data are presented as the mean and the SEM. Groups are compared using the Mann-Whitney U-test (controls and VNS group) or the Wilcoxon signed rank test (paired observations: day 7 and day 14). $P< 0.05$ was assumed to indicate a significant difference.

3. Results

3.1 Experiment 1: acute VNS

On the first day of the acute VNS experiment animals (n= 8) received 23 ± 5 stimulations, on the second day 29 ± 7 stimulations were given (NS). When the experiment was performed for the first time, the mean duration of the SWDs was higher when VNS was switched on (19.4 ± 1.7 s) compared to the baseline condition (13.3 ± 1.4 s) ($p< 0.05$, Figure 1). The second day of the experiment, the small difference in mean duration of the SWDs:

15.7 ± 2.8 s under baseline conditions versus 16.8 ± 1.8 s with VNS switched on, did not reach significance.

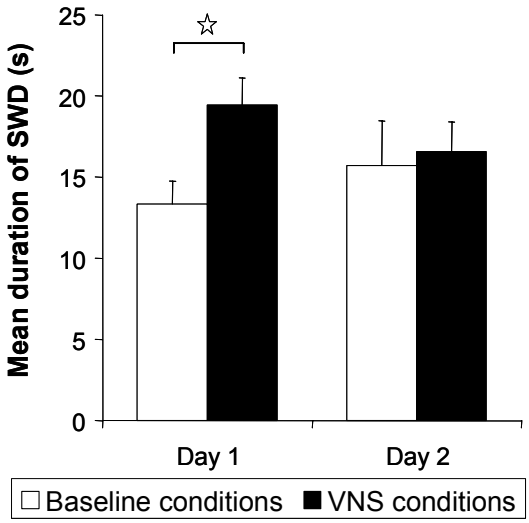

Figure 1: Experiment 1: Acute vagus nerve stimulation (VNS). Mean duration of SWD in GAERS (n= 8), without (open bars) and with (filled bars) the initiation of acute VNS once the SWD was detected. Identical experiments were performed on day 1 and day 2. Vertical lines on the bars indicate SEM. * indicates p< 0.05.

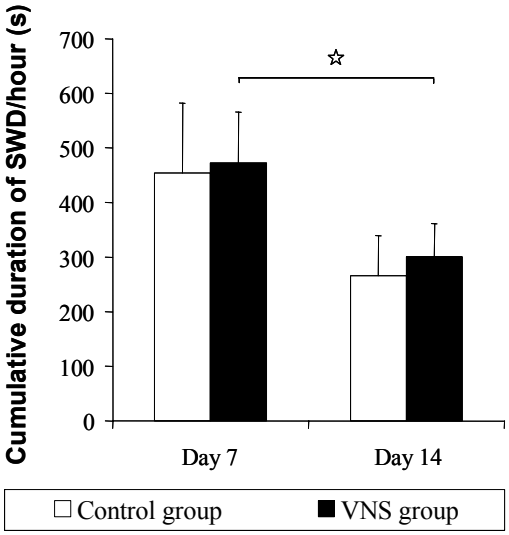

Figure 2: Experiment 2: Chronic vagus nerve stimulation (VNS). Cumulative duration of SWD in seconds over one hour of recording for GAERS without (n= 13) and with (n= 18) the application of the chronic VNS protocol (0.5 ms, 1.5 mA, pulses at 30 Hz, 60 s on, 12 s off). Vertical lines on the bars indicate SEM. * indicates p< 0.05.

3.2 Experiment 2: chronic VNS

SWD characteristics (number per hour, mean duration, cumulative duration per hour and peak frequency) of controls (n= 13) were compared with those of stimulated GAERS (n= 18). No differences in any of these parameters between control and treated animals could be detected (Table 1, Figure 2).

A decrease in cumulative duration of SWDs between baseline condition (day 7) versus stimulation condition (day 14) was observed in both the control group (n= 13) and the VNS group (n= 18) (Figure 2). This cumulative duration of SWDs/hour in the VNS group was significantly higher before (day 7): 472 ± 94 s, than after (day 14) one week of VNS: 300 ± 61 s (Figure 2). The other SWD characteristics were not significantly different between both conditions (Table 1).

4. Discussion

Basic research of VNS in convulsive generalized and partial epilepsy models (Lockard et al., 1990; Woodbury and Woodbury, 1990; Woodbury and Woodbury, 1991; Zabara, 1992; McLachlan et al., 1993; Takaya et al., 1996; Fernandez-Guardiola et al., 1999)

predicted positive results of VNS in epilepsy patients, but has so far failed to clearly identify good responders. Such fundamental data are presently completely lacking for absence epilepsy, a benign form of epilepsy. Human data are limited because VNS is only applicable in refractory absence epilepsy or when unacceptable side effects of anti-epileptic drugs (AEDs) occur. Investigating the efficacy of VNS in GAERS, a chronic epilepsy model with spontaneous seizures, could help to identify clues that predict the outcome of VNS. In this study we investigated the efficacy of VNS in a chronic animal model of epilepsy (seizures evolving from true and genetically-driven epileptogenesis) different from the common acute seizure models used so far, such as the PTZ model and the maximal electroshock model.

In experiment 1, a prolongation of the SWDs was found when VNS was started about 2.5 seconds after the initiation of the SWDs. An initial synchronization of epileptiform spikes and spike-wave activity on the EEG during VNS has been reported in man (Koo, 2001), which might resemble the initial increase in mean SWD duration observed here. We do not have a simple explanation for the aggravating effect of VNS on SWDs. At least it indicates that VNS stimulation can quickly exert an effect on local neuronal circuitry. On the other hand it also shows that SWDs are not easily aborted by VNS once they are ongoing. Absence seizures are non-convulsive and they most likely use different neuronal mechanisms than do convulsive events (Eskazan et al., 2002). Besides anecdotal reports of seizure aggravation by acute VNS, this is the first situation where VNS systematically prolongs an epileptic event, a phenomenon not uncommon for anti-epileptic drugs such as carbamazepine, phenytoin and $GABA_A$ mimetics (Danober et al., 1998).

The prolongation seems related to an acute effect of VNS; it disappears in a subacute experimental setting with several hours of VNS per day (Dedeurwaerdere et al., 2004) and also in the chronic setting of the second experiment. A difference between the effect of acute and continuous subthalamic nucleus (STN) stimulation on SWDs in GAERS has also been reported (Verceuil et al., 1998). SWDs could be aborted by acute STN stimulation, while there was no effect during a 10-min stimulation period.

VNS in man is typically delivered on the left vagus nerve. Krahl et al. (2003) have stated that right VNS is as effective as left VNS and should not induce significant cardiac effects when using clinically relevant stimulation parameters and a careful surgical technique. Stimulating both vagus nerves may affect a larger part of the brain and have a stronger effect on neuroexcitability, but this hypothesis has not yet been investigated. In the case of trigeminal nerve stimulation (TNS), bilateral TNS was more effective at reducing PTZ induced seizures than unilateral stimulation (Fanselow et al., 2000).

Table 2: Experiment 2: Chronic vagus nerve stimulation (VNS).

		Number of SWDs/h	Mean duration of SWDs (s)	Peak frequency of SWDs (Hz)
Control group (n= 13)	Day 7	37.0 ± 8.4	11.6 ± 1.7	8.1 ± 0.2
	Day 14	27.5 ± 6.4	9.6 ± 1.6	7.7 ± 0.2
VNS group (n= 18)	Day 7	35.7 ± 5.3	11.4 ± 1.2	8.0 ± 0.1
	Day 14	33.2 ± 5.0	8.5 ± 0.9	7.9 ± 0.1

Number over one hour of recording, mean duration and peak frequency of SWDs in control (n= 13) and chronic VNS group (n= 18) on day 7 and day 14.

In the chronic VNS experiment, cumulative SWD duration was significantly reduced in the VNS group, but the effect was very similar in the control group where it just did not reach significance. A previously reported spontaneous reduction of seizures in GAERS may be an interfering factor (Dufour et al., 2001; Rigoulot et al., 2003). This phenomenon is correlated with the decrease of vigilance resulting in more sleep episodes and less and shorter seizures (Dufour et al., 2001; Rigoulot et al., 2003). It could also explain the fewer seizures and shorter mean SWD durations that we found in our chronic experiment (Table 1) compared to previously published experiments in GAERS, which were monitored only for a short period of time (Danober et al., 1998).

Epileptogenesis in GAERS is a process that takes months, so it cannot be excluded that a longer VNS period is required to affect an already established absence epilepsy syndrome. This assumption is supported by several studies in humans describing a cumulative effect of VNS resulting in increased efficacy over a longer period of time (Vonck et al., 1999).

From this study we can conclude that acute and chronic (during one week) VNS have no suppressing effect on SWDs in GAERS. Moreover, acute VNS applied shortly after the onset of SWDs prolongs the mean duration of SWDs in GAERS at least during the first day of VNS.

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Vagus nerve stimulation does not affect memory in Fast rats, but has both pro-convulsive and anti-convulsive effects on amygdala kindled seizures.

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Submitted

Rationale: Using a seizure-prone Fast-kindling rat strain with known comorbid behavioral features, we investigated the effects of vagus nerve stimulation (VNS) on spatial memory, epileptogenesis, kindled seizures and body weight.
Methods: Electrodes were implanted in both amygdalae and around the left vagus nerve of 17 rats. After recovery, rats were first tested in the Morris water-maze (MWM) utilizing a fixed platform paradigm. The VNS group received 2 h of stimulation prior to entering the MWM. To investigate the effects of VNS on the kindling process, the VNS group received 2 h of stimulation prior to daily kindling stimulation. The abortive effects of acute VNS were then determined in fully kindled rats by applying VNS immediately after the kindling pulse. Body weight, water consumption and food intake were measured throughout.
Results: Memory performance in the MWM was not different between control and VNS rats. Also kindling rate was unaffected by antecedent VNS; besides, pro-convulsive effects were noted during the stage-5 convulsions in the VNS group ($p < 0.01$). Still, VNS showed anti-convulsant effects ($p < 0.01$) in fully kindled rats when applied immediately after the kindling stimulus. Body weight was significantly lower during kindling ($p < 0.01$) in VNS-treated rats compared to controls, which was associated with reduced food intake, but without difference in water consumption.
Conclusion: VNS stimulation appears to be devoid of significant cognitive side effects in the MWM. Although VNS exhibited no prophylactic anti-epileptic effect on epileptogenesis or seizure severity when applied prior to the kindling stimulus, it showed significant anti-convulsant effects in fully kindled rats when applied after seizure initiation. Lastly, VNS slowed the weight gain associated with kindling by reducing food intake.

Key words: vagus nerve stimulation (VNS), amygdala kindling, fast rats, memory, body weight

1. Introduction

Vagus nerve stimulation (VNS) is an efficacious broad-spectrum add-on treatment for patients with medically or surgically refractory epilepsy (Ben-Menachem, 2002). Treatment involves stimulation of the left vagus nerve via an implanted pulse generator in an attempt to reduce the frequency and severity of epileptic seizures. Controlled randomized trials with VNS have shown a 50% reduction in overall seizure frequency in approximately 30% of patients

(Salinsky, 2003). However, the mechanism of action remains unclear and basic research using chronic animal models of epilepsy is limited.

Kindling is considered to be one of the best animal models of temporal lobe epilepsy. Using a Fast-kindling rat strain, with known behavioral comorbidities, we investigated the therapeutic profile of VNS. The original Fast-kindling and Slow-kindling rat strains were created through selective breeding of two outbred parent strains (Long Evans Hooded and Wistar rats) (McIntyre et al., 1999). The selected strains demonstrate strong genetic control over amygdala epileptogenesis with faster rates of kindling in the Fast-kindling strain (Racine et al., 1999). In addition to faster kindling rates, the Fast strain shows behavioral comorbidities, including relatively greater impulsivity, hyperactivity and learning impairments compared to Slow rats (Anisman and McIntyre, 2002). Fast rats also exhibit greater body weights throughout development than the Slow strain.

Several studies have observed improved alertness, behavior and mood following VNS treatment in patients with refractory epilepsy, independent of changes in seizure frequency (Elger et al., 2000; Harden et al., 2000; Harden, 2001; Kossoff and Pyzik, 2004). In rats, as well as in humans, Clark and colleagues found that VNS was able to enhance memory storage when applied acutely during memory consolidation, which was dependent on an inverted-U shaped function of the stimulation current (Clark et al., 1995; Clark et al., 1998; Clark et al., 1999). In contrast, chronic VNS application in a clinical setting did not affect cognitive performance in patients with epilepsy using standardized tests (Hoppe et al., 2001; Dodrill and Morris, 2001). As a further test of the effects of VNS on memory processes, we wanted to investigate the impact of VNS on spatial learning in the Morris water-maze (MWM), when applied prior to each learning session in seizure-prone Fast rats, which have natural learning problems.

Initial animal studies showed promising results for VNS, which reduced both seizures (Lockard et al., 1990; Woodbury and Woodbury, 1990; Woodbury and Woodbury, 1991; Zabara, 1992; Takaya et al., 1996) and interictal EEG abnormalities (McLachlan, 1993). Consequently, VNS was further explored as a treatment for human epilepsy. VNS efficacy in animals has primarily been assessed in acute models (3-mercaptopropionate, pentylenetetrazole, maximal electroshock, penicillin or strychnine application) utilizing stimulation protocols immediately before and/or after seizure induction. However, only a few studies have assessed the effect of VNS in chronic animal models of epilepsy (Lockard et al., 1990; Munana et al., 2002; Dedeurwaerdere et al., 2004; Dedeurwaerdere et al., 2005). For instance, Fernandez-Guardiola et al. (1999) demonstrated that VNS inhibited epileptogenic processes during kindling in cats. Further investigation using such chronic epilepsy models may help to elucidate the mechanism of action of VNS, which should ultimately lead to a more hypothesis-driven approach to VNS clinical trials and guide the search for optimal

stimulation parameters and appropriate patient populations. This study was designed to investigate the effect of VNS on the progression of daily amygdala kindling in the Fast-kindling rat strain and to then examine its anti-convulsant properties in fully kindled Fast rats.

In addition, since weight reduction has been reported in patients treated with VNS (Burneo et al., 2002; Kneedy-Cayem et al., 2002), we wanted to validate this observation in Fast rats. In a previous study using genetic absence epilepsy rats from Strasbourg (GAERS), we observed a significant weight reduction after two weeks of chronic VNS (Dedeurwaerdere et al., 2003). Because seizure-prone Fast rats exhibit increased body weight compared to seizure-resistant Slow rats and amygdale kindling is known to increase body weight (Loscher et al., 2003; Bhatt et al., 2004), we aimed to study the effects of VNS on body weight throughout this study.

2. Methods

2.1 Animals

Seventeen Fast-kindling rats were bred and raised in the Life Science Animal Facility of Carleton University and treated in accordance with the guidelines of the Canadian Council on Animal Care and a protocol approved by the Carleton University Animal Care Committee. After weaning, rats were housed in pairs in standard opaque plastic cages (32 cm x 22 cm x 20 cm) with free access to food and water. They were maintained on a 12 h light/dark cycle (lights on at 8 a.m.) at 23°C with 60% relative humidity. At the time of the experiments, rats weighed between 300 and 450 g and were singly housed.

2.2. Surgery

After 4-7 days of daily handling, rats were anesthetized with sodium pentobarbital (Somnotol, 65 mg/kg) via intraperitoneal (i.p.) injection. A deep level of anesthesia during the surgical procedures was maintained by means of inhalation anesthesia utilizing Isoflurane (1%).

2.2.1. Implantation of the vagus nerve cuff-electrode

A self-sizing spiral silicone cuff-electrode (inner diameter: 0.7 mm) with recessed platinum contacts (surface: 1 x 2 mm, stimulation poles separated by 2 mm) and with flexible leads was used to deliver VNS. We have previously assessed the electrophysiological properties of this electrode and it was able to activate different nerve fibers at intensities lower than when using a simple wire cuff-electrode, likely due to minimized shunting of current to surrounding tissue.

The stimulation cuff-electrode was implanted as described previously (Takaya et al., 1996; Dedeurwaerdere et al., 2004). The cuff-electrode leads, attached to male Amphenol

pins, were tunneled subcutaneously towards the head and were inserted together with the EEG electrodes in a plastic head cap (Molino and McIntyre 1972).

2.2.2. Implantation of the depth electrodes

Bipolar stimulating/recording electrodes, consisting of two twisted strands of 0.127 mm diameter Diamel-insulated Nichrome wire attached to male Amphenol pins and cut to approximately 9 mm, were implanted in both amygdalae. Stereotaxic coordinates for the implantation were 0.2 mm posterior to bregma, 4.5 mm lateral to the midline and 8.2 mm below the skull surface (Paxinos and Watson, 1998). Five stainless steel screws, embedded in the skull and secured with dental acrylic, were used to anchor the electrodes in place. The Amphenol pins were inserted in the plastic head cap and secured in place by dental acrylic according to standard procedures (Molino and McIntyre, 1972). Following surgery, rats were given acetaminophen rectal gel, a topical analgesic (bupivacaine) and a subcutaneous injection of saline (NaCl 0.9%, 2 ml/kg) and then placed in plastic cages under warming lamps to maintain normal body temperature until behaviorally active. Rats were then returned to the colony room and allowed a two week recovery period before the initiation of kindling.

2.3. Vagus nerve stimulation

VNS was performed by connecting the cuff-electrode to an external NeuroCybernetic Prosthesis device (NCP, model 100; Cyberonics Inc., USA). Similar to VNS practice, the output current was ramped up to a just tolerable level of stimulation (Handforth et al., 1998). The following stimulation parameters were used for chronic stimulation: *output current*: 0.25-0.5 mA; *pulse duration*: 500 µs; *frequency*: 30 Hz; and *on/off duty cycle*: 30 s/ 1.1 min. When a stimulus of higher intensity was given, animals behaviorally reacted by flattening the ears, taking a fixed posture and sometimes vocalizing. A short test stimulus (*output current*: 1 mA; *pulse duration*: 500 µs; *frequency*: 30 Hz and *duration*: 1s), which evoked a behavioral response, was delivered daily in treated animals to check the integrity and impedance of the electrode-to-vagus nerve interface. The impedance values were always between 10 and 20 kΩ, which was within the range to deliver output currents of 0.5 mA.

2.4. Experiments

2.4.1. Effect of VNS on spatial memory in the Morris water-maze

The Morris water maze (MWM) is frequently employed to assess working memory. Much is known about the behavior of Fast rats in that context (Anisman and McIntyre, 2002). The MWM we used was a white, circular polypropylene pool (158 cm in diameter, 60 cm in height) that was filled with water (21°C and 38 cm deep) made opaque by the addition of powdered milk. A clear Plexiglas platform (14 cm in diameter) was submerged 2 cm below

the surface. The laboratory contained many extra maze cues for spatial orientation and the experimenter remained in the same position throughout the training.

The MWM test requires that an animal, placed in different start positions in the maze, locates and ascends a submerged platform. Acquisition performances were compared between control (n= 10) and VNS (n= 7) groups. All rats were placed in white plexiglass cages for 2 h before training began in the MWM, but only the VNS group received 2 h of stimulation, using the parameters described above. There were four training days during which the rats received four trials per day in the MWM to learn the location of the submerged platform and latency to reach the platform was measured. The platform remained in the same location throughout the entire experiment, while the starting location for the rats on the four daily trials varied between the four maze quadrants.

Cumulative latency to ascend the platform per day (sum of the four trials on each day) and per trial (sum of each trial over the four days) was calculated. Differences between the control and VNS groups in cumulative latencies were assessed using ANOVA with repeated measures. Data are presented as mean ± standard error of the mean (SEM) and significance is set at $p< 0.05$.

2.4.2. Effect of VNS on amygdala kindling

- **Effect of VNS on afterdischarge threshold (ADT)**

On the first day before daily kindling, initial (prekindling) ADTs and associated afterdischarges (AD) were determined for both amygdalae. The ADT was defined as the minimum stimulus intensity required to trigger a clearly discriminable, high-voltage, electrographic seizure event (an AD) that outlasted the stimulus by two or more seconds (Kelly et al., 1999). To assess the threshold, a 1 s, 60 Hz sine wave stimulus of progressively increasing intensity (15, 25, 35, 50, 75, 100, 150, 200, 300, 400, 500, 600 μA) was delivered until an AD was observed using a constant current generator. The interval between stimulations was 1 min. The interval between amygdala threshold determinations in the structure to be kindled (primary site) and contralateral amygdala was 10 min. During the ADT determinations, none of the animals received VNS. In one rat an AD could not be elicited and it was excluded from further kindling experiments. Post-kindling ADTs and associated behavioral profiles were also determined in both amygdalae 24 h after the last stage-5 behavioral seizure was elicited from the primary site.

- **Effect of VNS on kindling development**

Fast rats (n= 16) were stimulated in the amygdala once daily at their individual ADT intensity (Racine, 1972). During the course of kindling, if the rat failed to respond with an AD, it was stimulated at a higher intensity until an AD was evoked (using the increment technique described for ADT determination). Kindling continued until five stage-5 seizures

were accumulated. Kindling rate was defined as the number of AD-evoking stimulations necessary to develop the first stage-5 convulsion (Racine, 1972). Cumulative AD duration (ADD) was defined as the sum of ADDs from the primary site elicited prior to reaching the first stage-5 seizure. The stages of behavioral seizures were recorded and seizure severity was classified according to Racine (1972): Stage 1, immobility, eye closure, twitching of vibrissae, sniffing, facial clonus; Stage 2, head nodding associated with more severe facial clonus; Stage 3, clonus of one forelimb; Stage 4, rearing, often accompanied by bilateral forelimb clonus; Stage 5, rearing with loss of balance and falling accompanied by generalized clonic seizures. The convulsion profile of the five stage-5 seizures was determined by measuring latency to rearing and bilateral forelimb clonus, the duration of the stage-5 clonus and ipsilateral and contralateral ADDs .

During the kindling process, the vagus nerve was stimulated for 2 h prior to the kindling stimulus in the VNS group (n= 7), because it has been previously demonstrated that VNS has prophylactic action after prolonged stimulation (Takaya et al., 1996). In one VNS rat, electrical lead fracture occurred after four kindling days with VNS; hence this rat was excluded from the analysis. The control group (n= 8) was kindled in the same way as the VNS group, but did not receive VNS.

- **Anti-convulsive effect of VNS on stage-5 seizures**

In six animals with intact cuff-electrodes, the effects of acute VNS (for 60 s initiated using a magnet to trigger the device) on their stage-5 seizure profile was assessed. This VNS stimulation was applied approximately 2 s after induction of the kindling stimulus and the profile of the seizure was determined. Ten ‘control’ rats received the same kindling stimulation, without VNS. The next day, all rats received an additional kindling stimulation, which was also applied without VNS, as a further control observation. To assess the effect of acute VNS on amygdala kindled seizures, the following parameters were determined for all seizures: latency to bilateral forelimb clonus, the duration of clonus and the associated AD duration in the ipsilateral and contralateral amygdalae. In fully kindled rats, a stage-5 generalized convulsive seizure, characterized by forelimb clonus with rearing and falling, is generally evoked at the ADT. Thus, we also compared post-kindling ADTs and associated behavioral profiles between VNS-treated and control rats.

- **Statistical analysis**

Differences between control and VNS groups were determined using the Mann-Whitney U test for seizure severity (behavioral score) or by the Student's t-test for all other dependent values. ADTs and their primary ADDs at both the primary and contralateral sites were also compared before and after kindling using the paired Student's t-test. To determine differences in the convulsion profile between both groups, ANOVA with repeated measures

was used. Data are presented as mean ± standard error of the mean (SEM) and significance is set at $p < 0.05$.

2.4.3. Effect of VNS on body weight

During the kindling experiments, all rats were weighed daily. On four consecutive days, food and water consumption were also measured in control and VNS groups by weighing the water bottles and the food. Differences in weight gain during the kindling process (body weight on the day of prekindling ADT determination was used as reference), water consumption and food intake were assessed using ANOVA with repeated measurements.

2.5. Histology

Rats were perfused 24 h following the final kindling stimulus for histological assessment of stimulating/recording electrode placements. Each rat was deeply anesthetized with 65 mg/kg sodium pentobarbital and was then perfused intracardially with saline followed by 10% formalin. One day later, the electrodes were removed from the cranium and the brains were excised and stored in 30% sucrose for at least three days before sectioning. Frozen sections of 40 μm were taken through the electrode tract tips and stained with cresyl violet to identify the kindled site.

3. Results

3.1. Effect of VNS on spatial memory in the Morris water-maze test

Spatial memory was not altered in VNS Fast rats compared to control Fast rats in the MWM. Specifically, cumulative latencies to reach the platform over trials or days were not different between control and VNS groups (Figure 1). As expected, cumulative latencies to reach the fixed platform declined over days indicating a learning curve for both groups (Figure 1). Clearly, VNS stimulation had no negative impact on acquisition of learning in naïve seizure-prone Fast rats in the MWM.

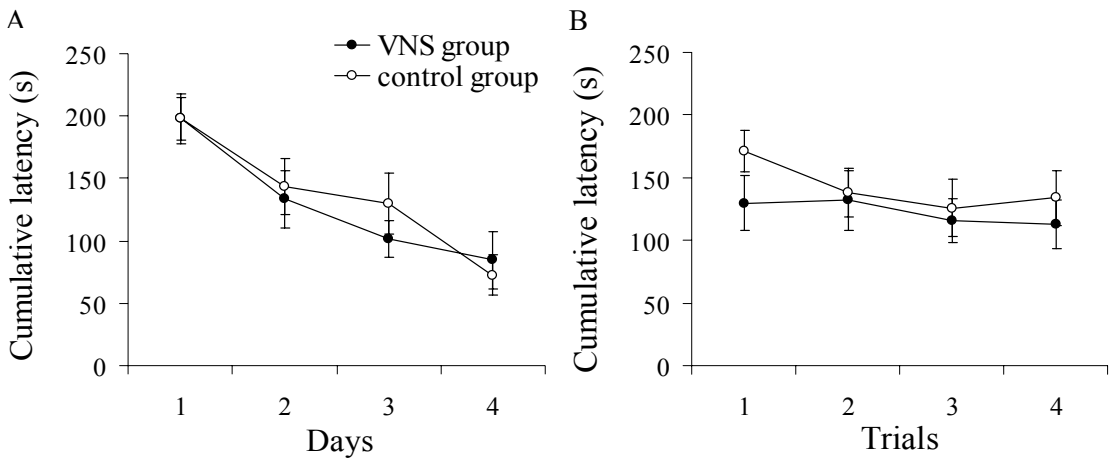

Figure 1: Effect of VNS on spatial memory as tested with a fixed platform position. Cumulative acquisition latencies to reach the platform for the control (n= 10) and VNS group (n= 7), A: on the four consecutive days and B: on the four daily trials. VNS was applied during 2 h before the Morris-water-maze test in the VNS group. Data are expressed as mean ± SEM.

3.2. Effect of VNS on amygdala kindling

In all animals, histological examination confirmed placement of the kindling electrodes in the amygdala.

3.2.1. Effect of VNS on afterdischarge threshold (ADT)

Before kindling, no significant differences were observed between VNS and control groups with respect to ADTs or evoked ADDs from either the ipsilateral or contralateral amygdala kindling sites (Table 1). After kindling, ADTs from the kindled and contralateral site were again not significantly different between the groups, yet ADT stimulation evoked a stage-5 seizure at the primary site in all previously VNS-treated rats, whereas the mean seizure stage evoked in control rats surprisingly was only 3 ± 1 ($p< 0.05$). This might suggest that previous VNS treatment can render the amygdala more excitable when VNS is not provided shortly before the ADT testing procedure.

As expected, ADDs were significantly prolonged in the amygdalae after kindling when ADTs were re-determined from both the primary (ipsilateral) and the contralateral site within the VNS group ($p< 0.01$, Table 1). This was also noted as a trend in the control group, but was however not significant ($p< 0.085$).

Table 1: Effect of chronic VNS on AD threshold (ADT) and AD duration (ADD) in the ipsilateral (= kindling site) and contralateral amygdala.

Group	N	Before kindling				After kindling					
		Ipsilateral site		Contralateral site		Ipsilateral site			Contralateral site		
		ADT (µA)	ADD (s)	ADT (µA)	ADD (s)	ADT (µA)	ADD (s)	Stage	ADT (µA)	ADD (s)	Stage
Control group	8	95 ± 19	27 ± 5	69 ± 20	16 ± 5	113 ± 16	54 ± 15	3 ± 1	75 ± 16	54 ± 11	0 ± 0
VNS group	7	101 ± 36	24 ± 3	62 ± 14	22 ± 5	89 ± 12	66 ± 10[a]	5 ± 0*	54 ± 12	74 ± 9[a]	1 ± 1

The mean (± SEM) ADT (µA) and its ADD (s) are presented before and after kindling in the control and VNS group. Before kindling, animals did not display behavioral seizures at ADT and the behavioral stages (± SEM) after kindling are presented in the table. None of the animals received VNS during the ADT determinations.

*Significantly different than the corresponding control group, $p < 0.05$.

[a]Significantly longer than the corresponding ADD before kindling, $p < 0.01$.

3.2.2. Effect of VNS on kindling development

Kindling rate and cumulative ADD to the first stage-5 seizure were not different between control (n= 8) and VNS treated (n= 7) rats (Table 2). Notably, one rat in the VNS group exhibited a kindling rate (23 stimuli) that was remarkably delayed in comparison to the control group (5.9 ± 0.5 stimuli).

Table 3: Effect of chronic VNS on kindling rate and cumulative afterdischarge duration (ADD) to develop stage-5 seizures in controls and VNS treated fast rats.

Group	N	Kindling rate	Cumulative ADD (s) to stage-5
Control group	8	5.9 ± 0.5	275.1 ± 36.7
VNS group	7	8.6 ± 2.5	502.0 ± 210.1

Control and VNS group were stimulated once daily at afterdischarge threshold (ADT). The VNS group received daily VNS for 2 h before kindling. The table shows the average number of stimulation sessions to the first stage-5 seizure and the cumulative ADD to the first stage-5 seizure (all data with SEM). Cumulative ADD at the primary site was calculated by summing up the individual ADDs until the first stage-5 seizure.

With respect to convulsion profile, no significant differences in latency to rearing with bilateral forelimb clonus were apparent between VNS and control groups (Table 3). Surprisingly, convulsion duration of the stage-5 seizures was actually longer in the VNS group than in the control group ($p < 0.01$, Table 3). Related to this, the mean ADDs of the ipsilateral and contralateral amygdala tended to be longer in the VNS group ($p < 0.09$), however not significantly.

Table 4: Effect of VNS on the convulsive profile (five stage-5 seizures) in amygdala kindled fast rats.

Group	N	Mean latency (s)	Mean convulsion duration (s)	Mean ADD (s) (ipsilateral)	Mean ADD (s) (contralateral)
Control group	8	20.3 ± 3.8	36.4 ± 4.1	76.6 ± 8.8	74.8 ± 9.2
VNS group	7	29.0 ± 7.2	56.9 ± 5.3**	103.6 ± 12.2	102.0 ± 12.6

All stimulations were carried out at ADT. Data are means and SEM of the five stage-5 seizures. Highly significant ($p < 0.01$) differences between both groups are indicated by **.

3.2.3. Anti-convulsive effect of VNS on stage-5 seizures

Statistically, when all VNS rats were treated equally, the VNS group showed significantly reduced ADDs in the ipsilateral and contralateral amygdalae in the anti-convulsive test ($p < 0.01$, Figure 2). Specifically, in two out of six VNS-treated rats, the stage-5 convulsion was completely suppressed when VNS was administered immediately after the kindling stimulus. Behavioral suppression in those two rats was associated with significantly

reduced ADDs in the ipsilateral and contralateral amygdalae. However, the other four VNS treated rats and all control rats (n= 10) displayed very similar generalized seizure profiles with respect to behavioral latencies, convulsion durations and ADDs. The next day, when VNS was withheld in the VNS group, no differences in behavioral stage, latency, convulsion duration or ADDs were evident between the two groups of rats (data not shown).

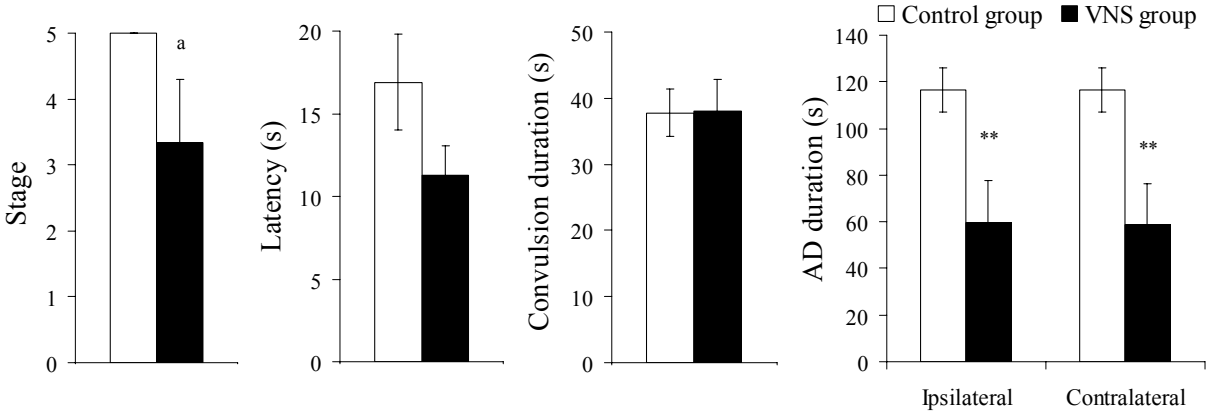

Figure 2: Anti-convulsive effect of acute VNS on stage-5 seizures in amygdala kindled Fast rats. After all animals had displayed five stage-5 seizures (fully kindled), anti-convulsive effects of VNS were tested by applying a 60 s VNS train immediately after the kindling stimulus was initiated in the VNS group (n= 6). Only the kindling pulse was administered to the control group (n= 10). Data are expressed as mean ± SEM, significance is set at $p< 0.05$, ** $p< 0.01$ and [a] $p= 0.059$ (borderline missed significance).

3.2.4. VNS side effects

During the kindling experiments, several side effects of daily VNS were observed, including increased urination and defecation, drooling and tearing (mostly left eye and sometimes both eyes).

3.3. Effect of VNS on body weight

Control Fast rats showed a gradual weight gain while the VNS group remained at a relatively stable weight throughout the kindling process (Figure 3). Hence, body weight was significantly ($p< 0.05$) lower in the VNS group throughout the majority of the kindling process. Accordingly, food consumption was significantly reduced in the VNS group compared to control Fast rats ($p< 0.05$, Figure 3). On the other hand, no difference in water consumption was observed between the two groups (data not shown).

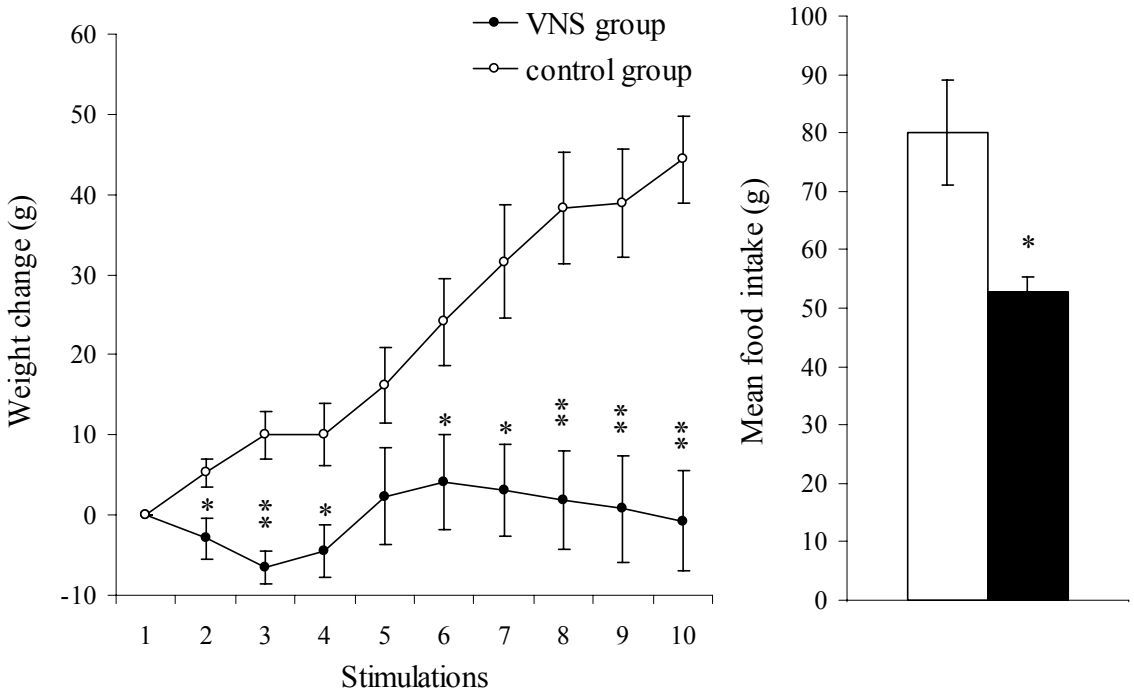

Figure 10: Effect of chronic VNS on body weight and food intake. The figure shows changes in body weight during the kindling experiments starting from the ADT determination until the 10th stimulation session. Body weight on the day of ADT determination (1st stimulation) was used as a reference point for each group. During four consecutive days water (not shown) and food intake (g) were measured. Data are expressed as mean ± SEM, significance is set at $p < 0.05$ and is indicated by an asterisk, * for $p < 0.05$ and ** for $p < 0.01$.

4. Discussion

The present study investigated the effect of VNS on spatial memory, development of amygdala kindling, the profile of generalized convulsions and weight gain in a highly seizure-prone rat strain (Fast rats) with natural comorbid learning impairments.

4.1. Effect of VNS on spatial memory in the Morris water-maze test

We found that applying VNS before daily training in the MWM did not alter acquisition of spatial memory of otherwise naïve Fast rats. Clearly, VNS did not negatively impact learning in this paradigm. While other therapeutic agents, especially AEDs, have been shown to exhibit mild to serious effects on cognition (Ortinski and Meador, 2004; Aldenkamp, 2001; Motamedi and Meador, 2004), multiple human studies have shown that VNS, using stimulation parameters typical of clinical settings, does not affect learning/memory (Hoppe et al., 2001). Similarly, we did not find changes in learning performance in the MWM following VNS treatment, which was in contrast to the results of Clark et al. (1995, 1998) who investigated rats in an avoidance test. Several factors may contribute to this discrepancy. We have applied VNS *prior* to the learning test, while Clark et

al. (1995, 1998), administered VNS *after* the test during memory consolidation. In addition, the stimulus current used in the various studies may also be relevant, since learning improvements in Clark's study were dependent on an inverted-U shaped function of the output current, with only intermediate currents being effective. The stimulation parameters used in our study, correspond to a strong rather than intermediate stimulation regimen. As described in the methods, just as is done in patients (Handforth et al., 1998), the output current was ramped up to the tolerance limit of the rat, which is the intensity believed to exert anti-epileptic effects.

4.2. Effect of VNS on amygdala kindling

An acute VNS train administered after the kindling stimulus in fully kindled Fast rats completely suppressed seizure severity in two of six rats and collectively shortened ADDs in our chronic amygdala kindling model of temporal lobe epilepsy. Other animal studies using acute seizure models have also observed this anti-convulsant effect of acute VNS (Woodbury and Woodbury, 1990; Woodbury and Woodbury, 1991; Zabara, 1992; McLachlan, 1993). In humans, 50-60% of patients who use the magnet to activate the VNS pulse generator benefit from magnet-induced VNS (Boon et al., 2001; Morris III, 2003). Our findings demonstrate VNS benefit in only a subpopulation of our Fast rats while other rats appeared relatively unaffected. Further research directed towards identification of critical criteria that leads to success for VNS application is warranted.

The mechanism through which acute VNS produces its anti-convulsant effect is not yet understood. However, some authors hypothesize possible involvement of non-specific arousal (Rutecki, 1990; McLachlan, 1993; Fanselow et al., 2000). For instance, McLachlan et al. (1993) observed that similar reductions in spike frequency were obtained by VNS application and by an arousing sensory stimulation treatment involving thermal stimulation of the tail. When acute VNS was applied at high intensity during absence seizures in GAERS, spike and wave discharges (SWDs) were stopped (Dedeurwaerdere et al. 2005). Indeed, absence seizures are often aborted by unexpected sensory stimuli (e.g. noise, touching the animal) in GAERS (Danober et al., 1998). On the other hand, when VNS was applied at a lower intensity, presumably not perceptible by the animal (indicated by no evidence of recognition), seizures were not interrupted by the stimulation and were even prolonged (Dedeurwaerdere et al., 2004, Dedeurwaerdere et al., 2005). Perhaps in a similar way, kindled seizures can be blocked by an antecedent footshock (Pinel et al., 1973). It has been demonstrated, in humans, that sensory stimulation can suppress focal spikes and absence seizures (Ricci et al., 1972; Rajna and Lona, 1989). Thus, beneficial effects from acute VNS could be mediated via nonspecific arousal, although other possible mechanisms of action cannot be excluded.

VNS did not affect kindling epileptogenesis in seizure-prone rats as evidenced by similar kindling rates and cumulative ADDs to the first stage-5 seizure in control and VNS groups. In apparent contrast, Fernandez-Guardiola et al. (1999) demonstrated that daily amygdala kindling (AK) was delayed in VNS treated cats. Stimulation parameters used in our study and the one of Fernandez-Guardiola et al. (1999) were similar, with the exception of the administration protocol. In the cat study, a VNS train of 60 s was applied before kindling and four times thereafter with a 60 min interval. Alternatively, a more likely cause for the discrepant findings would be the use of initially 'normal' cats versus our seizure-prone rats. It is conceivable that VNS cannot interfere with epileptogenesis in animals with a strong genetic predisposition to develop epilepsy, like the Fast rats in our study. Support for this hypothesis comes from a previous study in GAERS, a genetic model of absence epilepsy, wherein one week of VNS was not enough to decrease seizures significantly from control rats (Dedeurwaerdere et al., 2005). However, it is noted that in the present study we saw that some VNS-treated rats (n= 3) showed delayed kindling rates compared to controls (n= 3) when the piriform cortex, rather than the amygdala, served as the kindling focus (S. Dedeurwaerdere, personal observations, 2004). While kindling rate in the piriform cortex is normally much faster than in the amygdala (McIntyre et al., 1999), VNS rats did not display stage-5 seizures until VNS was terminated when kindled from the piriform cortex. Therefore, the origin of epileptogenesis could also be an important factor in the efficacy of VNS against kindling processes in Fast rats.

Two hours of VNS treatment administered prior to amygdala kindling stimulation in fully kindled Fast rats did not affect the latency to the stage-5 seizure between the two treatment groups. Surprisingly, however, convulsion duration was actually significantly longer in VNS-treated fully kindled animals. Similarly, the associated ADDs were also longer in the VNS group when VNS was applied for two hours before the kindling stimulus. Seizure aggravation is rarely reported in the clinical VNS literature (Koutroumanidis et al., 2000; Scherrmann et al., 2001; Connor et al., 2003); however increased synchronization of the EEG by VNS has been described (Koo, 2001). Further, Stoica and Tudor (1968) noted an increase in interictal spike frequency with high-voltage stimulation of the vagus nerve. In a previous study using GAERS, we observed a transient prolongation of SWDs associated with VNS treatment (Dedeurwaerdere et al., 2004; Dedeurwaerdere et al. 2005). The reports of seizure aggravation in humans have been anecdotally associated with higher output current (Koutroumanidis et al., 2000) and rapid cycling (Connor et al., 2003). However, it is generally assumed that increasing output current and duty cycle will improve efficacy (DeGiorgio et al., 2001). Nevertheless, evidence is available that VNS can have both inhibitory and excitatory effects in the brain.

Pre- and post-kindling ADTs were not significantly different between control and VNS rats. Ipsilaterally and contralaterally triggered ADDs were only significantly prolonged in the VNS group after kindling. After kindling, ipsilateral ADTs evoked stage-5 seizures in all VNS rats, which was not the case in the control group. Paralleling the human condition, it might be that if VNS therapy is discontinued after a prolonged period of treatment, there is a decreased capability to shut down or to control seizures. Accordingly, some patients with batteries reaching end of life, indicate increased seizure severity in comparison to periods before VNS treatment (K. Vonck pers. com.). Besides relapse of seizures, altered seizure patterns have also been reported (Tatum IV et al., 2004). In addition, it has been stated that postponing generator replacement may result in permanent loss of seizure control with VNS (Vonck et al., 2005).

4.2. Effect of VNS on body weight

Vagal afferents play a predominant role in the regulation of food intake (Schwartz, 2000). They carry signals from the stomach that pertain to the size and chemical composition of a meal to nucleus tractus solitarius, information that is then transmitted to satiety centers in the hypothalamus (Laskiewicz et al., 2004). Several studies performed in healthy animals found that vagal neuromodulation results in central and peripheral effects altering food intake and body weight down regulation (Roslin and Kurlan, 2001; Sobocki et al., 2001; Sobocki et al., 2002; Laskiewicz et al., 2004).

A basic association between amygdala kindled seizures and weight gain in rats has been documented (Loscher et al., 2003; Bhatt et al., 2004). In the present study we found that VNS treatment prevented the gradual increase in body weight observed in control rats throughout the kindling process. Interestingly, the amygdala has been suggested as a region where interactions between gustatory and vagal signs take place (Han et al., 2003). This reduction in amygdala kindling-induced weight gain in VNS rats was associated with reduced food intake compared to control kindled rats. In a previous study from our laboratory using GAERS, weight reduction was observed after two weeks of chronic VNS (Dedeurwaerdere et al., 2003). As in the kindling study the Fast rats only received two hours of VNS a day, it is possible that more continuous VNS would result not only in reduced weight gain, but also in reduced body weight as in GAERS. We are the first to report altered body weight in animal studies evaluating the efficacy of VNS on epilepsy likely because previous studies have primarily applied VNS acutely over very short time spans.

The effects of VNS on satiety and food intake could suggest an additional anti-convulsant mechanism of action of VNS by caloric restriction. Diets have been used for the treatment of intractable childhood epilepsy since the 1920s and are re-emerging as a treatment option (Kossoff, 2004), although the mechanism of action of these diets may be related to

induced ketosis. It has also been quoted that acute VNS could result in hemodynamically-induced hypoperfusion creating a relative deficit of energetic substrates (Sunderam et al., 2001).

5. Conclusion

In the present study we found that VNS with high-intensity stimulation parameters used in clinical settings does not affect spatial mapping memory in seizure-prone rats. When a VNS train was applied after the kindling pulse with the same stimulation parameters, stage-5 seizures could successfully be altered in some animals. However, VNS applied prior to the kindling stimulus was not able to prevent kindling epileptogenesis and had no prophylactic anti-convulsive effect on stage-5 seizures. In fact, seizure profiles in fully kindled Fast rats were worsened in the VNS-treated group. Finally, VNS treatment prevented weight gain associated with the kindling process presumably via the observed reduction in food intake. Clearly, the complexities of VNS treatment should be further investigated in order to optimize treatment in patients with refractory epilepsy.

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Chapter 5: Mechanism of action of vagus nerve stimulation

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The Mechanism of Action of Vagus Nerve Stimulation for Refractory Epilepsy

The Current Status

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Vagus nerve stimulation (VNS) is a neurophysiological treatment for patients with medically or surgically refractory epilepsy. Since the first human implant in 1989, more than 10 000 patients have been treated with VNS. The precise mechanism of action remains to be elucidated. Animal experiments with VNS were initially performed to demonstrate efficacy and safety preceding the clinical trials in human patients. Mechanism of action research involving animal experiments can provide essential clues. Animal experiments are often labor-intensive even in the hands of experienced researchers, however and the results remain only a reflection of the complicated pathophysiological systems of the human brain. Mechanism of action research in human patients treated with VNS is particularly challenging because of safety concerns, the large number of patients required and the heterogeneous nature of various small patient series. This study provides an overview of the progress that has been made in the past 10 years through neurophysiological, neuroanatomic, neurochemical and cerebral blood flow studies in animals and patients treated with VNS. Further elucidation of the mechanism of action of VNS may increase its clinical efficacy. It may also provide inspiration for the development of new therapeutic modalities for refractory epilepsy.

Key Words: Vagus nerve stimulation—Mechanism of action— Refractory epilepsy.

1. Introduction

When epileptic seizures are uncontrolled, diagnostic and therapeutic evaluation in a specialized setting is warranted. Alternative treatment modalities for patients with refractory epilepsy include trials with newly developed anti-epileptic drugs, epilepsy surgery and neurostimulation.

The introduction of a new anti-epileptic drug in the medication regimen of refractory patients results in seizure freedom in only a small percentage of patients (Fisher, 1993). Repeated inclusion in trials with novel anti-epileptic drugs may be associated with a low quality of life.

Epilepsy surgery is an alternative treatment for patients with medically refractory epilepsy in whom the “epileptogenic zone” can be identified and resected. Only 30% to 50%

of surgical candidates who are enrolled in the presurgical evaluation protocol eventually undergo surgery (Boon et al., 1999). For the remaining patients, there are few therapeutic options left.

The inability to provide adequate treatment for refractory patients with multifocal epilepsy provides an impetus to develop novel therapies. One such type of treatment is the electrical stimulation of nervous tissue to suppress seizure generation. Electrical stimulation of the vagus nerve is a neurophysiological treatment that was developed in the late 1980s and is currently available in specialized epilepsy centers worldwide (Handforth et al., 1998). Deep brain stimulation for epilepsy using electrical stimulation of areas within the brain is currently in the pilot study phase and is beyond the scope of this review.

The precise mechanism of action (MOA) of vagus nerve stimulation (VNS) and how it suppresses seizures remains to be elucidated. Most specialists in the field believe that greater insight in the MOA might help to identify certain seizure types or epileptic syndromes that respond better to VNS. It may also guide the search for optimal stimulation parameters leading to an increase in clinical efficacy. It may even lead to the development of new therapies (e.g., the identification of optimal stimulation targets for peripheral stimulation or deep brain stimulation). The purpose of this review is to provide an overview of the currently available data on MOA research in VNS.

2. History

The first vagus nerve stimulator was implanted in a human subject in 1989; however, the historical basis of peripheral stimulation for treating seizures dates back centuries. In the sixteenth and seventeenth centuries, physicians described the use of a ligature around the limb from which a seizure had started so as to arrest its progress. The method had been described many centuries before by Pelops, an ancient Greek author. He believed this observation was proof that epileptic fits originated from the limb itself. The hypothesis was reviewed in the beginning of the nineteenth century when Odier (1811) showed that ligatures were equally efficacious in arresting seizures caused by organic brain disease (e.g., a brain tumor). At the end of that century, Gowers (1881) attributed these findings to raised resistance in the sensory and motor nerve cells in the brain corresponding to the limb involved. This change of resistance, in turn, would arrest the spread of the epileptic discharge.

Gowers (1881) also reported several other ways by which sensory stimulation could prevent seizures from spreading (e.g., pinching of the skin and inhalation of ammonia). Almost a hundred years later, Rajna and Lona (1989) demonstrated that afferent sensory stimuli could abort epileptic paroxysms in human beings.

3. Anatomic basis

The vagus nerve is a mixed cranial nerve that consists of ~80% afferent fibers originating from the heart, aorta, lungs and gastrointestinal tract. Approximately 20% of these efferent fibers provide parasympathetic innervation of these structures and also innervate the voluntary striated muscles of the larynx and the pharynx (Agostini et al., 1957; Foley and DuBois, 1937; Paintal 1973). Cell bodies of the efferent fibers are located in the dorsal motor nucleus and nucleus ambiguous respectively. Afferent fibers have their origin in the nodose ganglion and primarily project to the nucleus of the solitary tract (NST). At the cervical level, the vagus nerve mainly consists of small-diameter unmyelinated C fibers (65% to 80%) and a smaller portion of intermediate-diameter myelinated B fibers and large-diameter myelinated A fibers. The NST has widespread projections to numerous areas in the forebrain as well as in the brainstem, including important areas for epileptogenesis such as the amygdala and thalamus. There are direct neural projections into the raphe nucleus, which is a major source of serotonergic neurons and indirect projections to the locus coeruleus and A5 nuclei which contain noradrenergic neurons. Finally, there are numerous diffuse cortical connections.

The diffuse pathways of the vagus nerve mediate important visceral reflexes such as coughing, vomiting, swallowing, control of blood pressure and heart rate. Heart rate is mostly influenced by the right vagus nerve, which has dense projections primarily to the atria of the heart (Saper et al., 1990).

4. Animal experiments

Early animal experiments investigated the effect of stimulation of the cervical vagus nerve on EEG. Depending on the level of anesthesia and the stimulus parameters used, VNS can induce EEG synchronization, EEG desynchronization, rapid eye movement and sleep or slow wave sleep in animals (Chase et al., 1966, 1967; Zanchetti et al., 1952). This was explained by the fact that different types of nerve fibers in the vagus nerve are activated when different stimulus parameters (frequency and intensity) are used. Desynchronization results from high-intensity and high-frequency (> 70 Hz) stimuli activating unmyelinated C fibers. Lower intensity high-frequency (> 70 Hz) stimulation induces synchronization because of activation of myelinated A and B fibers. Desynchronization may also be caused by high-intensity slower stimulation in the range of 20 to 50 Hz. Magnes et al. (1961) evaluated EEG changes induced by stimulation of the NST and found desynchronization when using frequencies above 30 Hz and synchronization when using a lower stimulation frequency in the range of 1 to 17 Hz. Because epileptic seizures are characterized by a paroxysmal abnormal synchronicity of EEG, it was believed that VNS could suppress seizures by desynchronizing the EEG.

Following up on this idea, the effect of VNS on clinical behavior and EEG epileptic activity in different animal models was studied. Vagus nerve stimulation blocks interictal spike activity induced by strychnine applied to the cortex of the cat (Zanchetti et al., 1952). Zabara (1992) found that generalized seizures in dogs induced by pentylenetetrazole and strychnine were inhibited by VNS and he made estimation about optimal stimulation parameters. These were found to be 20 to 30 Hz in frequency, 3.5 to 7 mA in output current and 0.2 ms in stimulus duration (Zabara, 1992). Woodbury and Woodbury (1990) established the anti-convulsant efficacy of VNS using frequencies greater than 4 Hz in rats after induction of seizures with pentylenetetrazole, 3-mercaptoproprionate and maximal electroshock. According to their research, the anti-convulsant effect is directly related to the fraction of unmyelinated C fibers stimulated. Chronic VNS also reduced the frequency of recurrent spontaneous seizures in monkeys with alumina gel foci (Lockard et al., 1990).

The results of these animal studies led to the development of an implantable device for human use and the first human trials for the treatment of epilepsy. Since 1989, more than 10 000 patients have been treated with VNS. This growing number of patients has aroused further interest in MOA research.

Other work has concentrated on the identification of anatomic brain structures that are potential mediators of the anti-seizure effect of VNS. Using c-*fos*, a nuclear protein that is expressed in neurons as a result of high neuronal activity, Naritoku et al. (1995) were the first to identify some key structures in the neuronal network between the brainstem and forebrain that are activated during VNS. Vagus nerve stimulation induced severe staining in limbic structures such as the amygdala, a highly epileptogenic region that plays a role in the generalization of seizures. The habenula and posteromedian nucleus of the thalamus, structures implicated in seizure regulation, also showed intense c-*fos* immunoreactivity. Support for a role of monoamines in the MOA of VNS was found through VNS-induced c-*fos* activation in the locus coeruleus and A5 nuclei of the brainstem. These two noradrenergic nuclei receive indirect projections from the nucleus tractus solitarius, the main efferent nucleus for the vagus nerve. Vagus nerve stimulation was shown to produce bilateral symmetric labeling in the previously mentioned brain structures, supporting the existence of a bilateral anti-convulsant effect despite unilateral left-sided stimulation. Because c-*fos* is a protein that signals transcription of other genes, VNS efficacy may be based on long-term neuronal changes. This hypothesis was confirmed by Takaya et al. (1996), who showed that the anti-seizure effect of VNS outlasts the duration of the stimulation train and that repetition of stimuli increases VNS efficacy. Inspired by the results from earlier studies on the circuitry involved in the anti-nociceptive effect and seizure-modulating effect of the locus coeruleus, Krahl et al. (1998) performed bilateral lesioning of the locus coeruleus in rats. As a result, seizure-suppressing effects of VNS in these animals were lost when treated with convulsant

chemicals. The authors claimed that lesioning of the locus coeruleus blocks the anti-convulsant effects of VNS by preventing VNS-induced norepinephrine release either globally or in some specific brain site.

Further investigation of neurotransmission on a neurochemical level was performed by Walker et al. (1999). These authors tried to establish the seizure-regulating influence of medullary nuclei by further investigating the role of gamma-aminobutyric acid (GABA) and glutamate synaptic transmission within the NST with respect to regulation of susceptibility to seizures of forebrain origin (Walker et al., 1999). They concluded that an increase in GABA or a decrease in glutamate transmission in the rat NST reduces the severity of limbic seizures. Vagus nerve stimulation may therefore exert its anti-seizure effect by inhibition of ascending outputs from the NST that project via various anatomic connections to the forebrain.

The early hypothesis of cortical desynchronization induced by activation of unmyelinated afferent vagal C fibers through the reticular activating system was further investigated by three different research groups. In human beings, effective therapeutic stimulation parameters are subthreshold for C fibers. Krahl et al. (2001) found strong evidence that vagal C fibers are neither responsible nor necessary for the seizure-suppressing effect of VNS. According to their experiments in awake and freely moving rats, activation of myelinated A and B fibers was shown to be responsible for seizure suppression.

Zagon and Kemeny (2000) performed animal studies at a cellular level. They particularly looked at the effects of the “weak stimuli” that predominantly activate the myelinated vagal afferents and are produced in cortical pyramidal neurons of the parietal cortex. They confirmed the findings by Krahl et al. (2001) on the role of myelinated afferent vagal fibers. Moreover, they showed that trains of vagal stimuli lead to prominent slow hyperpolarization of pyramidal cell membranes, reducing excitability. According to these findings, VNS is more likely to suppress neuronal excitability in cortical neurons, which may contribute to epileptic discharges, than to interfere with the synchronization of ongoing neuronal activity. Vagus nerve stimulation at low intensity repeated at such a rate that a second inhibition is initiated before the end of the previous inhibitory response may achieve sustained inhibition of the activity of involved cortical neurons.

Fanselow et al. (2000) investigated the effect of trigeminal nerve stimulation on seizures. They showed that stimulation of the infraorbital branch of the trigeminal nerve effectively reduces pentylenetetrazole-induced seizures in awake rats. Effective stimulation frequency (100 to 333 Hz) and current (9 to 11 mA) were higher when compared with parameters used in VNS. They believe that stimulation of cranial nerves suppresses seizures by activating the midbrain reticular formation and that this activation results in general arousal via the reticular activating system.

The efficacy and mode of action of VNS have mainly been studied in acute animal

models of epilepsy. Fernandez-Guardiola et al. (1999) performed VNS studies in electrically kindled cats, a model for chronic epilepsy. Their results showed that VNS delays the development of seizures induced by electrical kindling in the amygdala, suggesting a possible preventative effect of VNS on epileptogenesis. These results were confirmed by Naritoku and Mikels (1996, 1997), who evaluated the effects of VNS in electrically kindled rats.

To date, no results of studies in animal models with spontaneous primary generalized epilepsy have been published. Preliminary results from our group at Ghent University Hospital, who studied VNS efficacy in genetic absence epilepsy rats from Strasbourg, suggest that short-term VNS (3 h of stimulation, 1.5 mA, 30 Hz, 500 μs, 30 s on/5 min off during five consecutive days) does not reduce the frequency or duration of absence seizures (Dedeurwaerdere et al., 2004). Further studies using chronic stimulation need to be undertaken.

5. Research in human beings

The first experimental work in human beings aimed to reproduce EEG findings from animal experiments. Vagus nerve stimulation–induced suppression of interictal epileptic activity recorded by scalp EEG could not be demonstrated (Hammond et al., 1992a). In one patient, ictal EEG activity was abruptly terminated by acute VNS. In another patient, bilateral rhythmic δ activity during an aura was also interrupted by acute VNS. In three other patients, acute VNS delivered well into an ongoing seizure did not have any influence on the EEG or behavioral symptoms. Using fast-Fourier EEG analysis, no changes of normal background EEG during wakefulness, sleep or anesthesia were found.

Another study assessed the acute effects of VNS on background EEG activity using magnet mode activation (Salinsky and Burchiel, 1993). Epochs of 3 min of EEG were analyzed: 60 s before and after a VNS stimulation train and 60 s between during stimulation. Quantitative analysis did not reveal any changes in EEG background activity.

Other neurophysiological studies investigated VNS-induced vagal nerve evoked potentials (EPs) and the interference of VNS with clinically used EPs for diagnostic reasons. Vagus nerve stimulation induces a high-amplitude EP with a widespread scalp distribution (Hammond et al., 1992b). In one patient who was given a muscle-paralyzing agent during anesthesia, the vagus nerve EP was abolished. This suggested the presence of a myogenic EP rather than a true cortical EP, which was believed to be generated through activation of the recurrent laryngeal nerve. In this study, acute or chronic VNS did not have any influence on the different diagnostic EPs (visual, auditory brainstem, auditory 40 Hz and long-latency cognitive).

Naritoku et al. (1992) performed brainstem auditory EPs and somatosensory EPs to determine if chronic VNS results in electrophysiologically measurable changes. There was no

influence on brainstem auditory EPs. There was a significant increase in the interval between cervicomedullary and thalamocortical potentials (N13 to N20 interval) when three VNS patients were compared after one month of stimulation with three normal individuals and with baseline findings before VNS. An increase of somatosensory EP interpeak latencies has also been described in patients treated with phenytoin and carbamazepine. The prolongation of the interpeak latency may be related to the anti-epileptic effect of VNS. According to these authors, their findings support the hypothesis that VNS has effects on neuronal mechanisms that extend beyond structures immediately associated with the vagus nerve. The number of responders was too small (two responders) to allow meaningful conclusions on the prognostic value of these EP findings. The findings by Naritoku et al. (1992) were confirmed in another study that showed delays in latencies of evoked responses induced by direct esophageal stimulation in VNS-treated patients (Tougas et al., 1993).

Neurochemical studies quantified amino acid and neurotransmitter metabolite concentrations in cerebrospinal fluid (CSF) samples before and after VNS to clarify the hypothesis that VNS might act through the release of neurotransmitters and other compounds at the projection site of the vagus nerve (Ben-Menachem et al., 1995; Hammond et al., 1992c). The first study revealed selective increases in 5-hydroxyindoleacetic acid and homovanillic acid, metabolites of serotonin and dopamine, respectively. Also, a significant decrease in the levels of aspartate was found, correlating with better seizure control. The second study revealed increased GABA and ethanolamine levels. Although serotoninergic as well as dopaminergic systems have been found to have anti-convulsant effects in animal and human studies of various types of epilepsy, it remains to be clarified whether these findings are epiphenomena or findings directly related to VNS.

Finally, the effect of VNS on human central nervous system structures has been studied through cerebral blood flow (CBF) studies. Various changes in supratentorial and cerebellar CSF caused by acute and chronic VNS have been reported.

The first study was published by Garnett et al. (1992), who found ipsilateral activation in the thalamus and cingulate gyrus in five patients. A study by Ko et al. (1996) in three patients reported increased CBF in the left posterior cerebellum and putamen and in the right medial temporal gyrus and thalamus. The main conclusion was that VNS induces measurable CBF changes in anatomic structures that are part of the diffuse vagus nerve pathways. Occurrence of (subclinical) seizures during scanning and previous resective surgery in these early studies may have influenced the results and may account for discrepancies in the reported findings. Ko and his colleagues (1998) extended their original study and finally examined nine individuals in whom changes in CBF were correlated with seizure control. A reduction in seizure frequency best correlated with decreased CBF in the

right fusiform gyrus. Vagus nerve stimulation was also shown to exert an effect on CBF longer than the duration of the stimulation train.

The first positron emission tomography study of acute VNS showed diffuse CBF changes (Henry et al., 1998). Acute CBF changes were correlated with seizure control after three months of VNS (three responders versus 11 non-responders). Bilateral thalamic hyperperfusion correlated most significantly with a decrease in seizure frequency, suggesting that alterations in thalamic activity may contribute to anti-seizure effects of VNS. Repeated positron emission tomography studies in the same patients after three months showed that decreased CBF in bilateral hippocampi, the amygdala and the cingulate gyrus and increased bilateral insular CBF found during acute VNS was no longer present (Henry, 2000).

Our group performed an acute ^{99m}technetium single-photon emission computed tomography activation study in 11 patients receiving an initial stimulation train (0.25 to 0.50 mA, 30 Hz, 500 μs, 30 seconds) that revealed ipsilateral thalamic hypoperfusion as the most significant finding (Vonck et al., 2000). No significant CBF increases were found. In a follow-up study, 23 patients underwent an acute activation paradigm and a subgroup of 10 patients also underwent a single-photon emission computed tomography activation study after six months of chronic VNS. Cerebral blood flow changes were compared with clinical outcome (Van Laere et al., 2002). Chronic VNS (as compared to baseline CBF before stimulation) resulted in decreased CBF in bilateral thalami and the left caudate head. During the activation paradigm in a chronic situation in which the generator was activated with an additional 0.25 mA during 30 s, a significant left thalamic activation was found. An acute positron emission tomography study by the same group during the first stimulation train (0.25 to 0.50 mA, 30 Hz, 500 μs, 30 s) in six patients (Vandekerckhove et al., 2000) showed significantly increased CBF in the right thalamus, somatosensory cortex and left inferior cerebellum. Significantly decreased CBF was found in the left fusiform gyrus and in bilateral dorsolateral parietal cortex. In a recent study in eight patients, Ring et al. (2000) found bilateral thalamic perfusion decrease after chronic intermittent stimulation.

TABLE 1. *Most prominent findings in vagus nerve stimulation–induced cerebral blood flow changes*

Authors	Type of CBF change	Localization of CBF change	Lateralization of CBF change	Time of evaluation in VNS treatment	Type of study
Garnett et al.	↑	Thalamus	L	Chronic	PET
		Cingulate gyrus		Chronic	
Ko et al.	↑	Posterior cerebellum	L	Chronic	$H_2{}^{15}O$ PET
		Medial temporal gyrus	R	Chronic	
		Thalamus		Chronic	
	↓	Fusiform gyrus	R	Chronic	
Henry et al.	↑	Rostral medulla	B	Acute and chronic	$H_2{}^{15}O$ PET
		Hypothalamus		Acute and chronic	
		Thalamus		Chronic	
		Insular cortex		Acute	
		Orbitofrontal gyrus		Acute	
		Inferior parietal lobe		Acute and chronic	
		Inferior cerebellum		Acute and chronic	
		Thalamus	R	Acute	
		Postcentral gyrus		Acute and chronic	
		Entorhinal cortex		Acute	
		Temporal pole		Acute	
	↓	Amygdala	B	Acute	
		Hippocampus		Acute	
		Cingulate gyrus		Acute	
Vonck et al.	↓	Thalamus	L	Acute	^{99m}technetium SPECT
		Caudate head		Chronic	
		Hippocampus	R	Acute	
		Parahippocampal gyrus		Acute	
		Occipital cortex		Acute	
		Thalamus	B	Chronic	
Vandekerkhove et al.	↑	Inferior cerebellum	L	Acute	$C^{15}O_2$ PET
		Thalamus	R	Acute	
		Somatosensory cortex		Acute	
	↓	Fusiform gyrus	L	Acute	
		Dorsolateral parietal cortex	B	Acute	
Ring et al.	↓	Thalamus	B	Chronic	^{99m}technetium SPECT

CBF, cerebral blood flow; ↑, increase; ↓, decrease; L, left-sided; R, right-sided; B, bilateral; VNS, vagus nerve stimulation; PET, positron emission tomography; SPECT, single-photon emission computed tomography.

6. Discussion and conclusion

In the past 10 years, a large body of experimental literature on the MOA of VNS has been published. Research has focused mainly on four topics: neurophysiological (EEG and EPs), neuroanatomic, neurochemical and CBF studies. When comparing animal and human EEG studies, there is some concern about the discrepancies that were found. Although it was originally believed that desynchronization of the EEG induced by C fiber activation played a key role in the MOA of VNS, there are now strong arguments that this is not the case. The finding that VNS did not change interictal EEG activity in human beings as had been observed in animals was quite surprising. Moreover, human efficacy data from clinical trials show that stimulation at levels sub-threshold for activating C fibers is efficacious. Animal experiments further exploring this issue confirmed that C fibers most likely did not play a critical role. EEG recordings in animals were performed using epidural electrodes or depth electrodes. Such EEG recordings in human subjects are much less available. A single-case study with intracranial EEG during VNS was reported. Spectral analysis showed clear changes in α, β and γ power (Thompson et al., 1999). It is possible that visually undetectable subtle changes in scalp EEG occur during VNS. Quantitative techniques such as nonlinear dynamic analysis may help to clarify this matter.

The different level of consciousness of experimental animals in different studies also confounds interpretation of results. The earliest animal experiments were performed in anesthetized animals and differed from the studies by Krahl et al. (2001) in awake and freely moving animals.

Another point is that in the animal experiments, the effect of VNS was always evaluated when stimulation was performed in close relation to time of seizure onset, testing the anti-seizure effect of VNS. In human beings, VNS is used in an intermittent mode. Seizures are also suppressed when the stimulator is in the "off" mode, suggesting an anti-epileptic effect rather than an anti-seizure effect only. This finding is supported in the animal studies done by Takaya et al. (1996), who demonstrated that the efficacy of VNS outlasts the duration of the stimulation train. There may be different pathways involved in anti-seizure and anti-epileptic effects. Zagon and Kemeni (2000) suggested that chronic VNS suppresses neuronal excitability and induces a sustained hyperpolarization in cell membranes of cortical neurons.

Findings concerning the anti-seizure effect of VNS are concordant in animals and human subjects. When stimulated early enough in the course of an ictal EEG discharge (accompanied by behavioral symptoms), the seizure can be interrupted. This is also concordant with data from a magnet stimulation study that is reported elsewhere in this issue. Some authors suggest that this anti-seizure effect is strong enough in some patients to treat epilepsy, assuming that seizures might be predicted early enough to trigger stimulation of cranial nerves (Fanselow et al., 2000).

Concerning neuroanatomic structures that are important in the MOA, several authors have shown through different kind of studies that the locus coeruleus and the NST play a crucial role. Neurochemical studies support the hypothesis that these structures, when activated through electrical stimulation of the vagus nerve, may cause release of seizure-suppressing neurotransmitters.

Although CBF studies have contributed to the identification of brain structures that are located on the pathway from the cervical level of the vagus nerve to the cortex, their spatial resolution is not high enough to visualize these structures on an anatomic basis. A difference has to be made between structures on this pathway that may be activated during CBF activation studies and structures that truly play a role in the MOA of VNS. Varying results were found in different CBF studies, but the thalamus is most consistently involved. The VNS-induced CBF changes that were found suggest suppression of seizures by threshold modulation in structures known to play an important role in epileptogenicity. It is unclear whether activation of inhibitory neurons or an increase in inhibitory neurotransmitters is responsible for this.

Further studies in different animal models may reveal important clues about the MOA. Some initial interesting work has been performed in kindling models during the acute phase of kindling itself. Exploring the effect of long-term VNS in chronic seizures after kindling procedures have been performed may reveal even more interesting findings.

Vagus nerve stimulation is a patient-friendly treatment in the sense that it requires a minimally invasive procedure, has few side effects and requires minimum cooperation of the patient, which can be an advantage in mentally retarded patients. If proven not to be efficacious, the generator and lead can be removed successfully (Espinosa et al., 1999). Conversely, the palliative character and low efficacy of VNS compared with epilepsy surgery have to be considered. Further elucidation of the MOA may enhance clinical efficacy or identify structures within the brain that are potential targets for electrical stimulation.

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Small animal positron emission tomography during vagus nerve stimulation in rats: a pilot study

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Purpose: Vagus nerve stimulation (VNS) is an effective neurophysiological treatment for patients with refractory epilepsy, however, the mechanism of action remains unclear. Small animal positron emission tomography (PET) permits the monitoring of biochemical processes during multiple scans in the same animal. The aim of this pilot-study was to explore the potential of 2-[^{18}F]-fluoro-2-deoxy-D-glucose (FDG) PET to investigate the effect of acute and chronic VNS on glucose metabolism in the rat brain.
Methods: One week after surgery, a baseline FDG-PET scan was acquired during which animals were not stimulated. Secondly, scans were taken after first activation of the VNS electrode (acute VNS) and after one week of continuous VNS (chronic VNS). Images were obtained in a control group at the same time as the acute and chronic VNS scans. After acquisition, PET images were manually fused with MRI-data. Normalized brain activities and left/right activity ratios of different brain structures were compared between control measurements and VNS group.
Results: During acute VNS, glucose metabolism was significantly decreased in the left hippocampus ($p< 0.05$). Significant increases were found in both olfactory bulbs ($p< 0.05$).
A significant decrease in left/right ratio in the striatum ($p< 0.05$) was found which corresponded with a decrease in the left striatum, which was not significant ($p< 0.07$).
Conclusion: Acute and chronic VNS induced changes in glucose metabolism in regions important for seizure control (hippocampus, striatum). Our results are consistent with previous findings in patients with epilepsy using indirect perfusion markers.

Key words: small animal positron emission tomography (PET), vagus nerve stimulation (VNS), micro-PET, mechanism of action, rat, epilepsy.

1. Introduction

Vagus nerve stimulation (VNS) is used to reduce the frequency and severity of epileptic seizures in refractory patients, although the precise mechanism of action (MOA) still remains to be elucidated (Vonck et al., 2001). Understanding the MOA is an important aspect

of clinical VNS, as identification of responder groups and optimization of stimulation parameters is of great interest.

It is generally assumed that VNS exerts its effect by inducing action potentials in the afferents of the left vagus nerve, which has diffuse projections in the central nervous system. Several functional imaging studies have been conducted to investigate the activation or inhibition of brain structures by VNS. These studies found changes on both sides of the brain by unilateral left VNS and pointed out a key role for the thalamus and medial temporal lobe structures in the MOA of VNS (Vonck et al., 2001; Chae et al., 2003). However, there is no consensus on other activated structures neither on the type of changes (inhibition or excitation). This discrepancy can be attributed to a number of confounding factors such as imaging techniques used (PET, SPECT, fMRI), tracer and contrast agents, scanning protocols, stimulation parameters, medication regimes, course of the illness and treatment response. Heterogeneity of relatively small patient samples is difficult to avoid and, in addition, data gathering from healthy subjects is impossible for ethical reasons due to the invasiveness of VNS.

Small animal positron emission tomography (PET) is a technique used for in vivo, quantitative and high-resolution spatial determination of positron emitting isotopes in small animal tissues. It permits the monitoring of biochemical processes over time during a dynamic scan period but also longitudinally across multiple scans of the same subject. This technique is of increasing importance as fewer animals have to be used in comparison with post-mortem techniques like autoradiographic methods (Phelps, 2000). Small animal PET is widely used in all fields of pathophysiological research such as experimental oncology, gene expression and CNS research and is presently also emerging in animal research on epilepsy (Kornblum, et al., 2000). This technique could give the unique possibility to assess the effect of VNS on brain metabolism in healthy subjects, avoiding many of the disease-related confounding factors listed above.

Some of our previous imaging studies reported a different effect of acute and chronic VNS on cerebral blood flow, which is ascribed to a combination of an anti-seizure and anti-epileptic effect (Vonck et al., 2000; Van Laere et al., 2002). Therefore the aim of this study was to explore whether it is feasible to investigate the effect of acute and chronic effect of VNS on relative brain glucose metabolism in rats using micro-PET.

2. Materials and Methods

2.1 Animal preparation

Eight male Wistar rats, weighing 300-350 g, were used in this study. The animals were housed under environmentally controlled conditions (12 h light/dark cycles, 20-22°C) in the animal facility of the Ghent University Hospital with food and water ad libitum. All

animals were treated according to guidelines approved by the European Ethics Committee (decree 86/609/EEC). The study protocol was approved by the Animal Ethical Committee of Ghent University Hospital (ECP 01/26).

All animals were implanted with five epidural EEG electrodes and a stimulation cuff-electrode around the left vagus nerve under deep ketamine/xylazine anesthesia (80 mg/kg and 7.5 mg/kg respectively, i.p.) as previously described (Dedeurwaerdere et al., 2004). During surgery, additional ketamine (5 mg/kg) was given when sensorial pain prickles, by squeezing the foot pad, were felt. Following surgery, xylocaine (0.2 %) was applied at the incision wound and animals were placed under a heating lamp in their home cages to maintain normal body temperature until behaviorally active. The experimental period started at least one week after surgery and consisted of EEG recordings and repetitive scans.

2.2 EEG recordings

EEG was recorded during 3 h at the beginning and at the end of the experimental period to ensure that no spontaneous epileptic activity was present. For the EEG recording, a H2O™ portable digital 32-channel EEG recorder (Telefactor Corporation, USA) was used. The data were quantified by visual inspection of the EEG using TWIN™ off-line EEG analysis (Telefactor Corporation, USA).

2.3 Small animal PET imaging

The PET detector used for this study was the VUB-PET, developed at the University of Brussels (Belgium), which utilizes BaF_2 scintillation crystals coupled to a position and photo-sensitive wire chamber, containing Tetrakis-dimethylamine-ethylene (TMAE) vapor to detect annihilation photons (Bruyndonckx et al., 2001). The transaxial resolution at the centre was 2.6 mm and at 2.5 cm from the centre the radial and tangential resolution were respectively 4.3 mm and 3.8 mm. Details of the VUB-PET system have been discussed at length previously (Bruyndonckx et al., 2001; Tavernier et al., 1992) .

For the acquisition of the PET images, we used 2-deoxy-2[^{18}F]fluoro-D-glucose (FDG) as a tracer, which reflects regional glucose metabolism correlating with the degree of neuronal activity (Kornblum et al., 2000). FDG was synthesized by a previously reported method that is routinely used in our facility (Hamacher et al., 1968). FDG (11.1-14.8 MBq, 200 µl in volume) was administered i.v. through the dorsal penile vein under halothane gas anesthesia (5% induction and 1-1.5% for maintenance); thereafter the animals were placed fully awake in a dark, quiet room for 40 min. During this period, FDG is trapped in cells after phosphorylation by hexokinase, in a distribution reflective for conscious metabolic activity (Jacobs and Cherry, 2001). Brain activity initially rises rapidly; the rate of increase then progressively slows until activity is approximately constant between 30 and 60 min (Shimoji

et al., 2004). Images, acquired 45 min after FDG-injection, predominately reflect glucose metabolism of the first 15 min of FDG uptake. Subsequently, animals were brought under light anesthesia (medetomidine: 0.1 mg/kg and ketamine: 5 mg/kg, i.p.) to immobilize them in a fixed prone position in the centre of the scanner. Then, static PET scans in the fast and rotationary VUB-PET mode were performed for 10 min. No absolute quantification using blood sampling was carried out. Thus, we have not calculated the absolute cerebral metabolic rates for glucose (CMRGlc).

Three scans were acquired in the VNS group; a first baseline scan (n= 5) was obtained when animals were not stimulated. Secondly, a scan was taken (n= 4), when VNS was activated immediately after FDG injection, to investigate the acute effect of VNS. Finally, to measure the chronic effect of VNS, animals were scanned after one week of VNS (n= 3). During the acute and chronic VNS scan, the animals were receiving VNS during the 45 min of FDG uptake postinjection and during the scan. To compare acute and chronic VNS scans with a control group scans were also taken in non-stimulated control animals (n= 3) at the same time as the acute and chronic scan in the VNS animals.

2.4 Vagus nerve stimulation

Stimulation was performed by connecting the cuff-electrode to an external NeuroCybernetic Prosthesis device (NCP, model 100; Cyberonics Inc., USA). The impedance of the electrode-to-vagus nerve interface was measured at the beginning and end of each experiment; impedance values were the same before and after the experiments for all animals and were always between 1 and 8 kΩ. The following stimulation parameters based on previous studies (Dedeurwaerdere et al., 2004) were used: amplitude: 1.5 mA; frequency: 30 Hz; pulse duration: 500 μs; on/off cycle: 30 s/ 5 min.

2.5 MRI-scan

A single male Wistar Rat (300 g) was anesthetized with medetomidine (0.1 mg/kg) and ketamine (5 mg/kg), i.p. for immobilization. MRI was performed on a clinical 1.5 T whole body system (Symphony, Siemens, Germany) and the rat was placed in a wrist coil. After scout images were obtained, a 3D MPRAGE (Magnetization Prepared Rapid Acquisition Gradient Echo) *T1*-weighted image of the rat brain was acquired (repetition time (TR)= 1100 ms, echo time (TE)= 4.45 ms, inversion time (TI)= 640, 176 x 256 matrix, slice thickness 0.9 mm, field of view (FOV)= 52 mm x 75 mm x 29 mm, taking 38 min 34 s). The obtained DICOM images were extended with empty voxels in order to yield square image slices before fusion to the micro-PET images.

2.6 Analysis of PET-data

Data were collected in list mode and reconstructed using 2-dimensional ordered-subset expectation maximization (2D-OSEM: 4 subsets; 2 iterations; 19 coronal slices; 128 x 128 matrix) utilizing in house software (Bruyndonckx et al., 2001). No postsmoothing filter was applied. Axial (coronal) MRI-slices of a rat brain were used to assign regions of interest (ROIs) per slice (14 slices) by means of a rat brain atlas (Paxinos and Watson, 1998). Eight areas were pointed out on each hemisphere: olfactory bulb, cerebral cortex, striatum, hippocampus, thalamus and hypothalamus, cerebellum, medulla oblongata and brainstem. PET and MRI images were manually co-registered (Figure 1) using the multimodality package of the HERMES software (HERMES, Nuclear Diagnostics, Sweden). ROIs were matched on the corresponding PET slices. In this way activity was measured for all ROIs in 14 different slices and a mean value of activity per pixel was calculated.

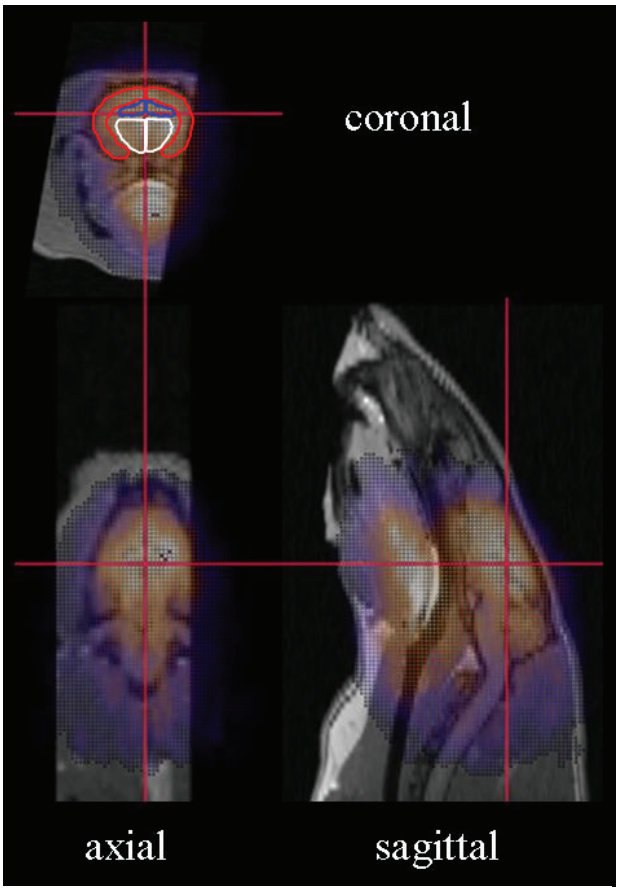

Figure 11: Co-registration of PET and MRI images. Coronal, axial and sagittal view of the rat brain. ROIs indicated in red: cerebral cortices, in blue: hippocampi, in white: thalami and hypothalami.

The Mann-Whitney U test was used to compare whole brain normalized activities (%) and left/right activity ratios of the different brain structures between control measurements and VNS group. As control measurements, normal data were taken together from both the direct control group as well as the baseline measurements from each set of animals (acute versus chronic VNS). Statistics were calculated with SPSS (v12 for windows, SPPS Inc, Belgium). Significance was set at $p < 0.05$ (two-tailed) and data are presented as mean ± SEM

3. Results

Ratios of glucose metabolism have been obtained in the regions of interest as mentioned above.

During acute VNS, significant changes in glucose metabolism were present in both olfactory bulbs (approximately 45% increased glucose uptake, $p< 0.05$) and in the left hippocampus (8% decreased glucose uptake, $p< 0.05$) (Figure 2). Metabolic values in the right hippocampus also tended to be decreased (11% decreased glucose uptake, $p< 0.09$) (Figure 2). No significant changes in left/right ratios were observed in the studied brain structures after acute VNS (data not shown).

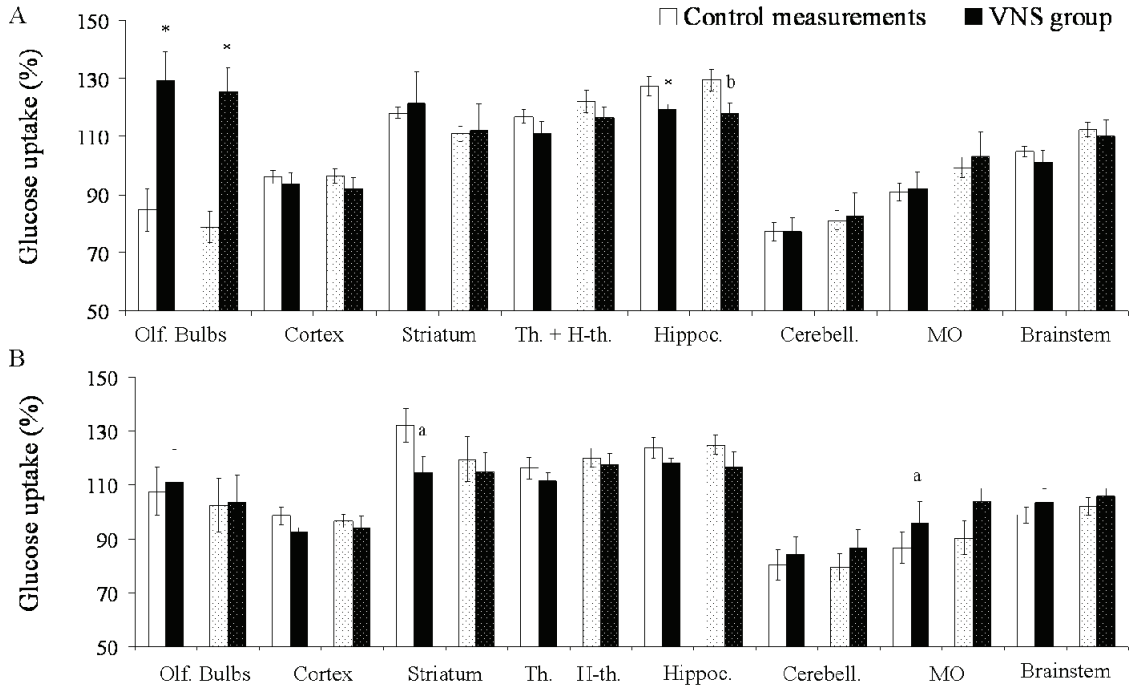

Figure 12: Effect of acute and chronic VNS on glucose metabolism in rats. Control measurements (white bars) were obtained without VNS. A. VNS was activated immediately after FDG-injection in the VNS group (black bars) and was on during the whole period of uptake and image acquisition. B. After one week of chronic VNS, additional images were acquired in the VNS group (black bars). Whole brain normalizations are shown for the eight brain regions. Plain bars are representing the left structure, dotted bars the right structure. Olf. Bulbs, olfactory bulbs; Th. + H-th., thalamus and hypothalamus; Hippoc., hippocampus; Cerebell., Cerebellum; MO, medulla oblongata. Data are expresses as mean ± SEM, significance is set at $p< 0.05$ and is indicated with an asterisk, a= $p< 0.07$ and b= $p< 0.09$.

During chronic VNS, there was a tendency for an increased glucose uptake of the left part of the medulla oblongata ($p< 0.07$). There was also a trend towards a decrease of the left striatum ($p< 0.07$), which corresponded with a significant ($p< 0.05$) decreased left/right ratio (1.00 ± 0.01) compared to control measurements (1.11 ± 0.01) (Figure 3).

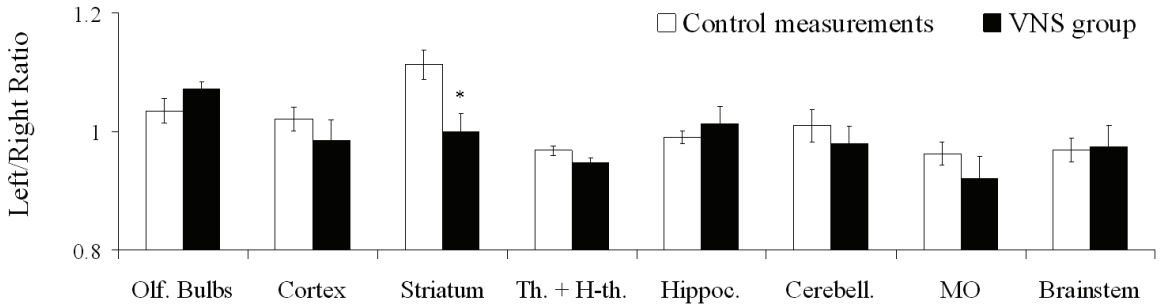

Figure 13: Effect of chronic VNS on left/right ratio of glucose uptake in rats. Control measurements (white bars) were obtained without VNS. After one week of chronic VNS, additional images were acquired in the VNS group (black bars). During the FDG uptake and image acquisition, VNS was still turned on. Left/right ratios are shown for eight brain regions. Abbreviations: Olf. Bulbs, olfactory bulbs; Th. + H-th., thalamus and hypothalamus; Hippoc., hippocampus; Cerebell., Cerebellum; MO, medulla oblongata. Data are expresses as mean ± SEM, significance is set at $p< 0.05$ and is indicated with an asterisk.

4. Discussion

We showed that it was feasible to measure changes in glucose metabolism during VNS using a small animal PET-detector and a limited number of rats. Acute VNS induced a significant decrease in left hippocampus and an increase of glucose metabolism in both olfactory bulbs. Chronic VNS significantly decreased left/right striatal ratios.

In this animal study, the results observed are in agreement with several previous imaging studies in epileptic patients (Henry et al., 1998; Henry, 2000; Henry et al., 2004; Van Laere et al., 2002; Vonck et al., 2000). We found a decrease in the left hippocampus (trend in the right hippocampus), a structure known to be often involved in the generation of complex partial seizures in temporal lobe epilepsy. A decreased glucose metabolism in this region may reflect the anti-convulsant activity of acute VNS. More specifically, in patients, acute VNS can be triggered at seizure onset by a magnet to interfere with the seizure. In line with the long-term study of Henry et al. (2004), these changes in hippocampal CBF were absent after chronic VNS. It is believed that these differences between acute and chronic VNS reflect cerebral adaptation to chronic VNS (Henry, 2000). This adaptation might underlie the finding that therapeutic VNS often causes gradual improved seizure control over several months of treatment (Henry, 2000).

In the current study, acute VNS bilaterally increased glucose uptake in the olfactory bulbs. Evoked potential and unit activity recording techniques have revealed the existence of a vagus nerve-olfactory bulb pathway (Garcia-Diaz et al., 1984). Also in patients, VNS has been found to influence and modulate the processing of olfactory information (Kirchner et al., 2004). This last neurophysiological study reported even a positive correlation between activation of the olfactory bulb and therapeutic benefit. As the olfactory bulb is on the

contrary very small in humans, it is conceivable that metabolic changes in this structure were not measurable in human imaging studies.

The increase of glucose metabolism in the left medulla oblongata after chronic VNS was not significant (P= 0.07) and a higher number of animals might be necessary to demonstrate a difference in this structure. Changes in metabolism in dorsal-rostral medulla during acute and chronic VNS have also been reported in CBF studies of Henry (2000). These findings correspond to the known anatomical connections and projections of the vagus nerve to the brainstem tracti and nuclei.

The striatum is part of the basal ganglia and consists of the caudate nucleus and putamen. It is fairly large (around 5 mm3) and well separated in the rat brain; because of this, changes can be successfully measured even at moderate 3-4 mm spatial resolution (Hume et al., 1996). During baseline measurements, glucose metabolism in the striatum was slightly higher on the left side than on the right side (ratio: 1.1). In one human study, this has also been observed in healthy males (Kawachi et al., 2002). After chronic VNS, we found a significant decreased left/right ratio in striatum in comparison with baseline and a tendency towards a decrease in the left striatum. This brain structure has been acknowledged to play an important role in the control of seizures (Deransart and Depaulis, 2002). Ictal hyperperfusion of the striatum was shown using single photon emission computed tomography (SPECT) in patients with temporal lobe epilepsy (Semah, 2002). The striatum receives indirect innervation from the vagus nerve via the medial reticular formation of the medulla which projects to the intralaminar nuclei of the thalamus (having extremely widespread projections to cerebral cortex and the striatum) (Henry, 2002). Moreover, in PET imaging studies in humans, changes in CBF have been found in the putamen due to VNS (Ko et al., 1996).

Our study and brain imaging studies in humans have reported changes in both hemispheres (Chae et al., 2003) confirming that unilateral left VNS has bilateral functional projections into the brain.

In this pilot-study we compared relative differences in glucose metabolism (whole brain normalizations and left/right ratios of brain structures) after acute and chronic VNS with control measurements. Comparison with specific reference structure such as the cerebellum was judged as impossible, because it is presently unknown how glucose metabolism in such a structure will be altered by VNS. An alternative approach to whole brain normalization could include the scanning of a phantom with known radioactivity together with the rat and to utilize brain structure/phantom ratios as dependent values. The use of relative quantitative values was preferred for several reasons. The performance of absolute quantitative longitudinal studies would require the placement of a chronic, indwelling arterial catheter, which would increase potential morbidity and mortality in the experimental animals (Kornblum et al., 2000). Recently, several studies are endeavoring to acquire absolute

quantification of CMRGlc necessitating measurement of plasma glucose levels and the setup of automated microsampling systems (Lapointe et al., 1999). Nevertheless, the blood sampling procedures during the FDG uptake period could affect the animals' behavior and thus the results (Kornblum et al., 2000). Moreover, the comparative ease of performing studies using relative (as opposed to absolute) quantization can increase small animal PET's accessibility to many investigators (Kornblum et al., 2000).

For more precise anatomical localization, autoradiography may provide functional data at higher spatial resolution than can be achieved with PET. Although much more animals are needed and comparison within the same animal is impossible, autoradiography is still the paramount method to detect submillimetre FDG changes in the rat brain. The resolution of the current VUB-PET system is limited, and therefore more subtle changes or changes in small structures can be missed due to the partial volume effect. Indeed, previously reported changes in both directions in thalamus (Henry et al., 1998; Vonck et al., 2000; Henry et al., 2004) and hypothalamus (Henry et al., 1998; Henry et al., 2004) (for review see Chae et al., 2003) were not observed in this study. The negative findings in these regions therefore do not exclude the presence of smaller or more localized changes and will need confirmation in a higher-resolution acquisition or analysis. Secondly, due to the presence of Harderian glands near the frontal cortex, significant inscatter effects cannot be excluded, although, no changes were measured in glucose uptake in the Harderian glands due to acute or chronic VNS. One way to optimize the analysis is to incorporate partial volume correction schemes based upon high-resolution anatomical information, which can be performed post-hoc (but then MRI scans for all individual animals are needed) or even partial-volume corrections during reconstruction (Baete et al., 2004).

In this study we have co-registered a single MRI template with PET images, which greatly improves the interpretation of the PET images. Instead of software fusion, integrated multimodality imaging systems e.g. combining PET with CT or MRI can obtain most accurate hardware fusion. Currently, hybrid systems like small animal PET/CT are being marketed and research is focusing on integrated (small animal) PET/MRI devices as well (Gaa et al., 2004).

In conclusion, we showed that small animal PET is a useful and promising technique for imaging of cerebral activation in long-term studies in rats. In this feasibility study only a limited group of animals were studied. In the future, it would be appropriate to further extend the study towards the effect of VNS on brain metabolism by using higher-resolution acquisition, individual MRI scans and increased numbers of animals. In addition, it would be of great interest to investigate VNS its properties in different animal models of epilepsy (e.g. genetic absence epilepsy models, status epilepticus models). These studies could be focusing on different stimulation parameters (high or low intensity stimulation), prolonged stimulation

over months, different tracers (e.g. [3H]-Flumazenil a cBZP/GABAA) or absolute CMRGlc. Briefly, this pilot study demonstrated with restricted means the potential of small animal PET as an explorative tool for studying rat brain metabolism and responses to treatments during several stages of VNS therapy.

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Chapter 6: General discussion

General discussion

Neuropharmacological therapy with levetiracetam (LEV) and vagus nerve stimulation (VNS) are two novel treatments for refractory epilepsy. Acute application of both treatment options can be very effective. LEV can act rapidly on seizures in both animals and humans. In addition, preclinical studies suggest that LEV may have anti-epileptogenic and neuroprotective effects, with the potential to slow or arrest disease progression. VNS too can have an immediate effect on seizures in animals and patients with in addition an important cumulative effect after prolonged treatment. These treatments can be considered as neuromodulatory, since there is evidence that changes in central nervous system function or organization are induced by influencing and initiating neurophysiological signals.

In this general discussion, we will deal with different aspects of these treatments with regard to the results found in this Ph.D. project as well as in the light of the current literature. Also future perspectives will be presented for both types of treatment.

6.1 Levetiracetam

Lately, several novel anti-epileptic drugs (AEDs) were added to the pharmacotherapeutic armamentarium. One of these drugs is LEV (ucb L059, "Keppra") with a wide spectrum of anti-convulsive effects in animal models for different types of epileptic seizures (Loscher and Honack, 1993; Klitgaard et al., 1998; Loscher et al., 2000). Apart from its anti-seizure effect, there is evidence that LEV also exhibits anti-epileptogenic properties in the amygdala kindling model of epilepsy (Loscher et al., 1998) and the spontaneously epileptic rat (SER), a model of primary generalized epilepsy (Sasa et al., 2003).

Administration of LEV suppresses the occurrence of absence seizures in genetic absence epilepsy rats from Strasbourg (GAERS), a validated model of absence epilepsy (Gower et al., 1995). The effect of LEV on the development of epilepsy in a model of primary generalized absence epilepsy has not been studied so far. In a pilot study in GAERS, the robust anti-seizure effect of LEV was confirmed and a trend towards an anti-epileptogenic effect was found (Boon et al., 2002). This encouraged us to further investigate the neuromodulatory properties of LEV in GAERS; it was felt that investigating the effect of chronic LEV treatment in young GAERS could provide new insights and strategies for the treatment of epilepsy. During this Ph.D. research, we have investigated the effect of LEV on the age-related development of spike and wave discharges (SWDs) in GAERS by chronic administration of LEV (postnatal day (PN) 23-PN60) starting before the age of occurrence of SWDs. The effect of early chronic LEV administration on the development of SWDs was evaluated in young GAERS during treatment (PN57-PN60), immediately after treatment termination (PN61-PN64) and two months after the last LEV injection (PN120, age four months), when brain maturation was achieved and SWDs recorded on cortical EEG were numerous.

We found that chronic LEV administration induced a decrease in epileptiform events (SWDs and "short irregular SWDs"= SISWDs) in young GAERS (PN57-PN60), which persisted to some extent immediately following cessation of treatment (PN61-PN64), but not until adulthood (PN120).

6.1.1 Clinical relevance

The treatment of epilepsy focuses exclusively on suppressing seizures, which is one of the end products of epilepsy. The question is whether epileptogenesis can be prevented or modulated by therapeutic intervention (anti-epileptogenesis). There are several possible clinical roles for anti-epileptogenic compounds (Schachter, 2002):

1. An anti-epileptogenic drug could prevent seizures in patients at high risk for developing epilepsy. This could include patients undergoing craniotomy, patients with stroke or

severe brain injury and patients with a family history of a genetically determined epilepsy syndrome.

2. An anti-epileptogenic therapy could induce permanent seizure remission in patients with epilepsy that would persist after the drug was discontinued.
3. An anti-epileptogenic drug could halt or reverse an increasing seizure frequency or severity in patients with progressive epilepsy syndromes.
4. If epileptogenesis could not be prevented or stopped, an anti-epileptogenic drug could modify the disease process in such way that epilepsy would become easier to treat.

As the underlying mechanisms of epileptogenesis are still largely unknown, pharmacological prophylaxis has no solid theoretical basis, but is predicted on the assumption that what is good for seizure control will also help to prevent the development of an epileptic focus. On the other hand, anti-epileptogenic drugs do not necessarily have an effect on the seizures themselves.

At present, human studies have not revealed an ideal strategy for anti-epileptogenesis. A number of AEDs have been evaluated in clinical trials to test whether they prevent epileptogenesis in humans, but to date no drug has been shown to be effective in such trials. But we also have to keep in mind that none of the new AEDs have yet been adequately tested for anti-epileptogenic effects in humans (Temkin, 2001). However, animal experiments could give some clues on anti-epileptogenic properties of AEDs.

6.1.2 Animal research on neuromodulatory and anti-epileptogenic properties of AEDs

A number of AEDs have been tested in the kindling model of epilepsy for a pharmacological prophylactic effect (Table 1). In this model, drugs can either be tested in fully kindled rats for their effect on elicited seizures (anti-convulsant effect) or drugs can be administered during the kindling process to test whether they suppress kindling development (thought to reflect an anti-epileptogenic effect). Various compounds have been found to interfere with the kindling process (Table 1) (Loscher, 2002).

Several studies have been performed in the status epilepticus (SE) models (Table 2). There are different treatment strategies in the timescale from the initial insults through the latent period followed by the actual onset of the seizures (Figure 1).

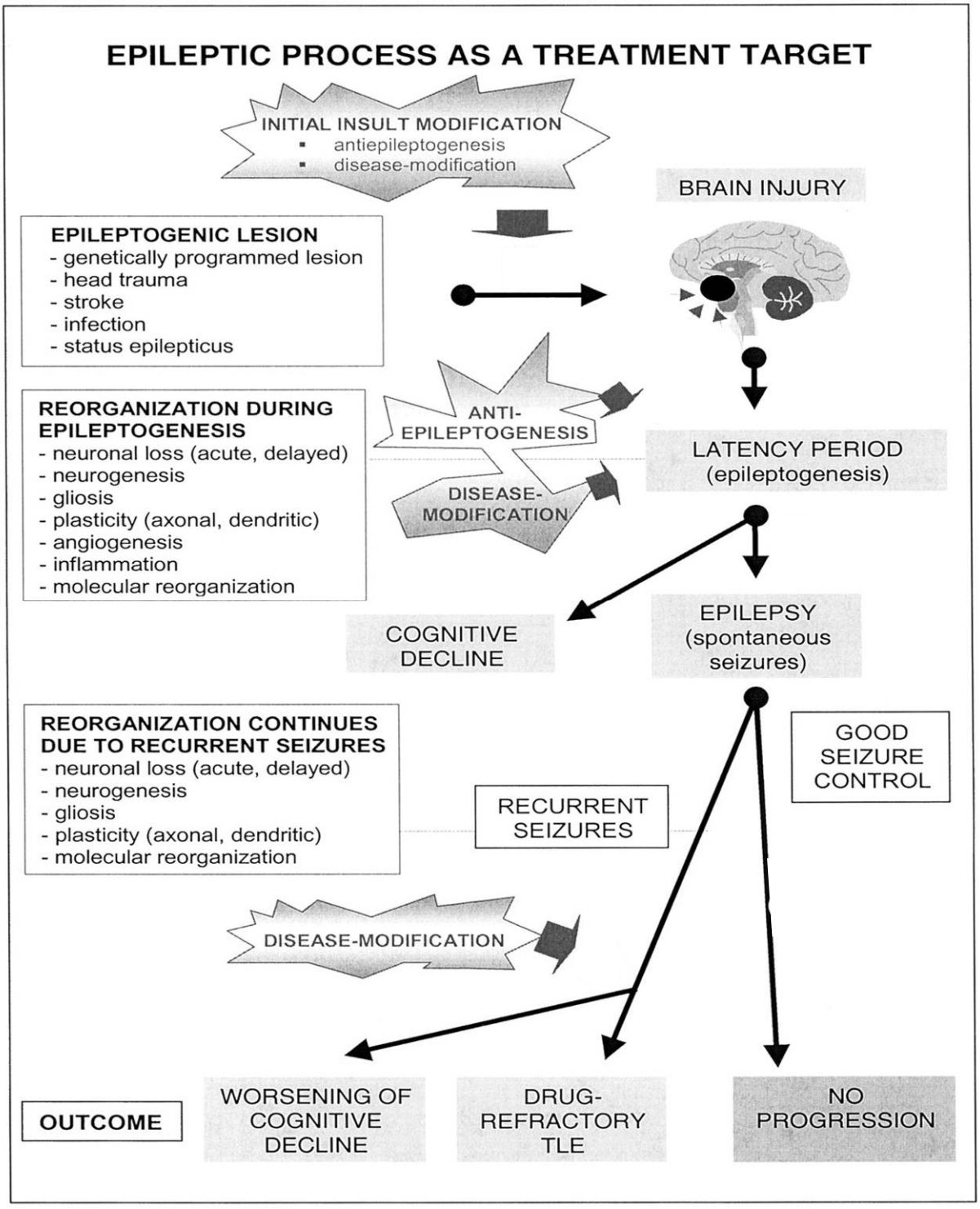

Figure 1: Epileptic processes as a treatment target during the development of acquired epilepsy (Pitkanen, 2002). Various etiologic factors, such as genetically programmed lesions, head trauma, stroke, infection and SE, can cause neuronal damage in the brain. Brain injury triggers epileptogenesis in a subpopulation of patients during which time different neurobiological changes occur. These include acute and delayed neuronal loss, neurogenesis, gliosis, axonal and dendritic plasticity, angiogenesis, inflammation and molecular reorganization of receptors and channels. This period provides a window of opportunity for anti-epileptogenic compounds that can either completely prevent the development of epilepsy or at least have disease-modifying effect. After the epileptogenic latency period, spontaneous recurrent seizures appear and epilepsy is diagnosed. In parallel to initial insult-induced epileptogenesis, cognitive performance might also decline. Good seizure control can be obtained in approximately 70% of the patients. In the remaining patients, molecular, cellular and network reorganization can continue even after the diagnosis of epilepsy because of recurrent seizures. This might have two functional consequences: worsening of epilepsy and further decline in cognitive functions.

Table 1: Effects of AEDs in the amygdala kindling model in rats (Loscher, 2002).

Drug	Suppression of kindling development	Suppression of fully kindled seizures
Valproate	+	+
Phenobarbital	+	+
Benzodiazepines	+	+
Vigabatrin	+	+
Topiramate	+	+
Levetiracetam	+	+
Lamotrigine	+	+
Carbamazepine	NE	+
Phenytoin	NE	+
NMDA antagonists	+	NE

An anti-kindling or anti-seizure effect is indicated by '+'; lack of such effect by 'NE', not effective.

In the SE models, prevention of epilepsy was seen with diazepam, levetiracetam, valproate and topiramate (Table 2). However, the anti-epileptogenic findings of levetiracetam, topiramate and valproate could not be reproduced in other SE-models (Klitgaard and Pitkanen, 2003).

Table 2: Overview of anti-epileptogenesis studies in SE models adapted from Loscher (2002).

Drug	Model	Prevention of epilepsy	Reduction of neurodegeneration	Reference
Carbamazepine	PPS	n.d.	NE	Halonen et al., 2001a
Caspase-inhibitors	Kainate	n.d.	(+)	Ebert et al., 2002
Diazepam	SAS	+	+	Pitkanen et al., 2005
Gabapentin	Kainate	n.d.	+	Cilio et al., 2001
Ketamine	Pilocarpine	NE	+	Hort et al., 1999
Lamotrigine	PPS	n.d.	+	Halonen et al., 2001a
Levetiracetam	Pilocarpine	NE	+	Klitgaard et al., 2001
Levetiracetam	PPS	+	n.d.	Mazarati et al., 2003
MK-801	Kainate	NE	+	Ebert et al., 2002
Phenobarbital	Kainate	NE	NE	Bolanos et al., 1998
Remacemide	PPS	n.d.	NE	Halonen et al., 1999
Retigabine	Kainate	n.d.	(+)	Ebert et al., 2002
Topiramate	Pilocarpine	+	+	DeLorenzo et al., 2002
Topiramate	SHS	n.d.	+	Niebauer and Gruenthal, 1999
Topiramate	SAS	NE	NE	Pitkanen, 2002
Valproate	Kainate	+	+	Bolanos et al., 1998
Valproate	Pilocarpine	NE	n.d.	Klitgaard and Pitkanen, 2003
Vigabatrin	SAS	NE	NE	Halonen et al., 2001b
Vigabatrin	Pilocarpine	NE	+	Andre et al., 2001
Vigabatrin	Kainate	n.d.	NE	Pitkanen et al., 1999

Only studies are shown in which drugs were given after a status of sufficient length to induce epilepsy and brain damage. An anti-epileptogenic or neuroprotective effect is indicated by '+', lack of such effects by 'NE', not effective; weak effect by '(+)'; not determined by 'n.d.'. Abbreviations: SAS, sustained amygdala stimulation; SHS, sustained hippocampal stimulation; PPS, perforant path stimulation.

Discrepancies between studies using the kindling model and studies using the SE model indicate that mechanisms underlying development of epilepsy in different animal models might not be the same (Loscher, 2002). In addition, there is a lack of data from other models of epileptogenesis and it is difficult to extrapolate the data of kindling and SE models to other epileptogenic etiologies such as stroke- or head trauma-induced epileptogenesis.

Animal models of genetic idiopathic epilepsy have only been used scarcely. Absence seizures, a type of idiopathic epilepsy in humans, are usually a benign form of epilepsy (Wolf, 1984). Despite the good pharmacological response in this form of epilepsy, investigating the anti-epileptogenic properties of AEDs in GAERS could provide new insights and strategies for epilepsy treatment. In GAERS, spontaneous absence seizures develop progressively. Therefore, it is an excellent model to study epileptogenesis and interference with epileptogenesis by neuromodulation. To our knowledge, there is only one study that chronically administered an AED i.e. remacemide in young GAERS (PN7-PN25) to establish its effect on SWD development (Nehlig and Boehrer, 2003). This study failed to demonstrate an effect of early remacemide treatment on the expression of SWDs in adult GAERS.

6.1.3 Animal research on levetiracetam

LEV has an anti-epileptic effect in a broad range of animal models mimicking both partial (Loscher and Honack, 1993; Klitgaard et al., 1998; Loscher et al., 2000; Glien et al., 2002) and generalized epilepsy in man (Loscher and Honack, 1993; Klitgaard et al., 1998) including GAERS (Gower et al., 1995). In contrast, LEV revealed no effect in the two conventional screening tests, namely the maximal electroshock and pentylenetetrazole models (Klitgaard et al., 1998; Loscher and Honack, 1993).

The anti-seizure effect of LEV has mainly been established after a single injection of LEV. However, in several studies LEV was administered during prolonged periods (Loscher et al., 1998; Loscher and Honack, 2000; Glien et al., 2002; Stratton et al., 2003). We found that chronic LEV treatment significantly suppressed (average difference of 62%) epileptiform events in young GAERS (PN57-PN60) in comparison with control animals (Dedeurwaerdere et al., 2005). LEV was administered daily by i.p. injection and its effect was investigated during treatment (3 h a day for four consecutive days) and immediately after termination of the treatment (3 h a day for four consecutive days) in young animals. Young GAERS have few SWDs; therefore the more frequent SWD-like events were also taken into account. These were defined as short irregular SWDs (SISWDs) with clear spike and wave aspects characterized by lower amplitude (< 3 times baseline amplitude) or lower peak frequency (5-7 Hz) and shorter in duration (< 2 s) (Dedeurwaerdere et al., 2005). The LEV treated animals displayed less SWD-like events (up to 81%) than the control group even in the pre-injection hours. This could suggest that there was a slowing down of the epileptogenic processes by

early chronic treatment (PN23-PN60) with LEV. Yet, the lower number in the pre-injection hours might also be caused by retention of LEV in the brain from previous injections, although LEV was already eliminated from the blood plasma.

Interestingly, there was no acute decrease in SISWDs after injection of LEV (PN57-PN60) (Dedeurwaerdere et al., 2005). This is in contrast with previous acute experiments in adult GAERS, in which a clear decrease in SWDs could be observed after a single LEV injection (Gower et al., 1995; Boon et al., 2002; Bouwman and van Rijn, 2004). These studies, which were performed in adult GAERS, report the effect of a first and single LEV injection, while the effect of chronic LEV administration was not investigated. Moreover, several studies dealing with the chronic administration of LEV in animal models of temporal lobe epilepsy report increased functional tolerance towards LEV and some loss of efficacy after prolonged administration (Loscher et al., 1998; Loscher and Honack, 2000; Glien et al., 2002). This increased tolerance could provide a possible reason for the lack of an acute decrease in SISWDs in GAERS that were already pretreated.

When treatment was stopped, there was no sudden increase in epileptiform events (SWDs and SISWDs) (Dedeurwaerdere et al., 2005). This is in line with previously performed rat and mouse experiments in which chronic treatment with LEV did not lead to withdrawal hyperexcitability (Klitgaard et al., 1998; Loscher and Honack, 2000). Moreover, there was a trend ($p= 0.064$) towards a lower number of epileptiform events (an average difference of 53%) in the young LEV pretreated GAERS. In the study of Glien et al. (2002) LEV was administered during two weeks in the pilocarpine model of temporal lobe epilepsy. Some animals still had less spontaneous partial seizures one week after terminating LEV treatment and this was described as a carryover effect (Glien et al., 2002).

Neuroprotective and anti-epileptogenic properties of LEV have been reported in several animal models of epilepsy. LEV induces neuroprotection in a rat model of focal cerebral ischemia (Hanon and Klitgaard, 2001). Recent observations showed that LEV attenuates both hippocampal cell death and enhancement in hippocampal excitability following a pilocarpine induced SE (Klitgaard et al., 2001). Another study demonstrated that chronic levetiracetam treatment has a mild neuroprotective effect on hippocampal principal cells and interneurons after pilocarpine-induced SE (Pitkanen and Kubova, 2004).

These neuroprotective properties may be relevant to its anti-epileptogenic action. Klitgaard et al. (2001) induced pilocarpine-induced SE in rats and terminated it with diazepam 30 min after the beginning of SE. Thereafter, levetiracetam treatment was initiated and continued for the next 21 days. Continuous video-EEG monitoring (72 h) three days after drug cessation revealed, however, no differences in the percentage of pretreated rats with spontaneous seizures compared to a vehicle group. Mazarati et al. (2003) started levetiracetam treatment 1, 3 and 6 h after PPS and continued it for 29 days via subcutaneous

osmotic minipumps. Two weeks after treatment discontinuation, seizures were analyzed using video-EEG monitoring. Seizure frequency was reduced and the number of seizure free days was greater in the levetiracetam group compared to the vehicle group.

In the amygdala kindling model, LEV suppressed kindling development at doses devoid of adverse effects with persistent reduction in afterdischarge duration after termination of treatment (Loscher et al., 1998). These findings have recently been confirmed (Stratton et al., 2003; Zhang et al., 2003). The ability of LEV to delay the development of kindling suggests that it has the potential to interfere with the epileptic circuitry modifying seizure recruitment and projection properties of the epileptic network (Loscher et al., 1998). However, LEV has been administered before the kindling stimulus was given. There are some controversies whether or not its anti-convulsant action might have prevented the development of kindling by reducing the expression of the evoked seizures rather than affecting the processes underlying the hyperexcitibility of the fully kindled state. We have put up a pilot study in which LEV (54 mg/kg i.p.) and/or valproate (200 mg/kg i.p.) was administered daily for 24 consecutive days before kindling (De Smedt et al., 2003). One week after the last injection, kindling procedures were applied according to the "rapid kindling with recurrent hippocampal seizures (RKRHS) model". Preliminary results did not show differences in kindling rate or seizures expression from the vehicle group.

The anti-epileptogenic potential of LEV has also been supported in the genetic SER model characterized by spontaneous tonic convulsions and absence seizures (Sasa et al., 2003). LEV was administered during weeks 5–9 after birth before the appearance of spontaneous seizures and was terminated at the expected age for seizure expression, which resulted in a decrease in the frequency of both tonic and absence seizures in pre-treated animals. Also, the duration of both tonic convulsions and absence seizures was shorter than in controls.

In a previous study, we found an indication for such an anti-epileptogenic effect in the GAERS model (Boon et al., 2002). Recently, we found that epileptiform events tended ($p= 0.064$) to be less frequent (up to 60%) in LEV pretreated (PN23-PN60) GAERS during the four observation days after treatment cessation in comparison with the control group (Dedeurwaerdere et al., 2005). The major metabolite of LEV is pharmacologically inactive and other metabolites are produced at levels below those that are relevant for pharmacological activities (Loscher et al., 1998). LEV does not interfere with enzyme systems (Patsalos, 2000; Benedetti, 2000) and is washed out rapidly (half-live ~2-3 h) after stopping the treatment (Loscher et al., 1998). The decrease in epileptiform events on the first two recording days after treatment cessation might be due to retention of LEV or other metabolites in the brain; however, this is unlikely for the last two recording days. Early chronic treatment with LEV in

young GAERS might have caused an initial slowing down of the epileptogenic processes, but had no effect on the later expression of SWDs (age of four months).

It is clear that LEV can have a lasting and sometimes even permanent neuromodulatory effect on the epileptic central nervous system. Chronic treatment is probably not just a summation of several single administrations of LEV. Additional phenomena appear like prolonged effect after treatment for days (Dedeurwaerdere et al., 2005), a week (Glien et al., 2002) and even permanent modifications of the epileptic network occur (Loscher et al., 1998). Nevertheless, undesired effects like functional tolerance have also been described after chronic treatment (Loscher and Honack, 2000; Zhang et al., 2003).

6.1.4 Mechanisms of action of levetiracetam

Until now, it is not described in what manner LEV exerts its effect on absence seizures in GAERS. A possible effect of LEV that might contribute to its action against SWDs in GAERS could be by progressively decreased firing of neurons of the substantia nigra pars reticulata (Loscher et al., 1996). A reduction in the firing rate of these neurons has been shown to result in anti-convulsant activity (Loscher and Ebert, 1996). In addition, direct micro-injection of LEV into the SNR induces seizure protection in the pilocarpine model of temporal lobe epilepsy (Klitgaard et al., 2003). The SNR is not part of the epileptic circuit underlying absence seizures, but it may belong to a system participating in a remote control over seizures (Danober et al., 1998). Indeed, high frequency stimulation of the SNR has been shown to interrupt SWDs in GAERS (Feddersen et al., 2004) and intracerebral micro-injections of GABA mimetics in the SNR suppress absence seizures (Danober et al., 1998). In line with this, Klitgaard et al. (1998, 2003) stresses that a part of LEV's ability to suppress seizures may originate from its effect on neuronal firing in the SNR, but the anatomical network and molecular mechanisms by which this is mediated remains to be demonstrated. We hypothesize that LEV's anti-epileptic action on SWDs in GAERS is mediated through an effect on the SNR, but it is not known if this might interfere with epileptogenesis. In GAERS pups, metabolic increases in the nigral inhibitory system such as SNR compared to non-epileptic control rats were observed (Nehlig et al., 1998). Increase in these structures involved in the control of seizure activity was suggested to reflect a mechanism of prevention of epileptogenesis in GAERS.

6.1.5 Future perspectives

As the incidence of new epilepsy cases is between 26 and 70 per 100 000 (Hauser, 1998), it would be favorable to not only search for treatments that suppress seizures, but also to seek for treatments that tackle the underlying pathophysiology of epilepsy itself. A new

approach to drug screening, including the process of epileptogenesis, may identify new classes of drugs (Kupferberg, 2001; Klitgaard and Pitkanen, 2003). LEV is a novel AED with potential anti-epileptogenic properties, which was discovered using non-conventional drug screening. Not only the properties of the drug itself, but also the time frame during which it is applied is of great importance. More research is required to determine the optimal time windows for preventing epileptogenesis in several animal models of epilepsy.

In the *SE model*, it has been shown by several investigators that the optimal time window to pharmacologically intervene with the initial insult (SE) is within 2 h (Brandt et al., 2003; Pitkanen et al., 2005). Pretreatment with LEV by i.v. injection via the tail vein reduced or prevented (higher doses) the development of self-sustaining seizures (Mazarati et al., 2004). Treatment during the maintenance phase of self-sustaining status epilepticus diminished or aborted seizures (higher doses) (Mazarati et al., 2004). The authors, however, did not mention whether these animals were further monitored to investigate the development of seizures and to assess the neural damage, which would have been of interest. In some studies this initial interference was extended by continuing treatment during the latent period (Klitgaard et al., 2001; Mazarati et al., 2003; Pitkanen and Kubova, 2004). This approach has been shown to induce mild neuroprotection and to interfere with epileptogenesis in the PSS induced SE model, but not in the pilocarpine SE model. It has not been assessed whether seizures are easier to treat when epilepsy was not prevented, but delayed or reduced in severity.

In the *amygdala kindling* model, LEV had anti-epileptogenic properties when applied before the kindling stimulus. In a pilot-study we performed, epileptogenesis was not altered when chronic LEV treatment was applied before the kindling procedure was started (De Smedt et al., 2003). Another interesting strategy would be to apply the AED immediately after the evoked seizure to interfere with processes underlying epilepsy development during kindling. In our laboratory, a new experimental design has been set up to further investigate the anti-epileptogenic properties of LEV on kindling development. An alternative of the RKRHS model, which does not lead to permanent hyperexcitibility (Lothman and Williamson, 1994) is the RKRHS model with alternate day kindling. In this model, kindling is performed every two days during eight days. During the subsequent week, epileptogenic processes are indelibly imprinted into the brain resulting in a permanent hyperexcitibility (Lothman and Williamson, 1994). The week of epileptogenesis can be regarded as a “latent period”, enabling to interact with epileptogenesis without interfering with the kindling seizures themselves (pers. com. T. De Smedt). If permanent hyperexcitibility is affected by treatment during this week, this would provide evidence of an anti-epileptogenic effect of the tested compound.

Animal models of genetic idiopathic epilepsy have only just been used in anti-epileptogenesis research. In *GAERS*, therapy was started (PN23) before seizures became apparent to interfere directly or indirectly with early pathological changes. Although there was a temporal effect of LEV on seizures immediately after treatment discontinuation, seizure expression was not altered during adulthood. It is known that important pathophysiological changes (T-type calcium conductance of reticular thalamic neurons, glucose metabolism, astrocytic modifications and glutamate transporters) in GAERS emerge early in life, before the appearance of spontaneous absence seizures (Dutuit et al., 2000; Dutuit et al., 2002; Tsakiridou et al., 1995; Vergnes et al., 2003). Some of these neuropathological and neurophysiological changes take place before we started the treatment in this study (Vergnes et al., 2003). Therefore, it could be possible that earlier initiation of LEV treatment, e.g. beginning at birth, could lead to a more sustained effect. The chronic delivery of LEV did not interfere with normal development and body weight of the animals. Clinical findings suggest that LEV is a well-tolerated and safe treatment option for pediatric patients with epilepsy (Patsalos, 2000; Coppola et al., 2004). Importantly, Bittigau et al. (2003) has, however, demonstrated that the same AEDs, including diazepam, clonazepam, phenytoin, phenobarbital, valproate and vigabatrin, can induce apoptotic neurodegeneration in the developing brain in clinically relevant doses. Pitkanen et al. (2004) commented that extrapolation of data obtained in mature brain to immature brain should be done with caution, hence, neuroprotection in the immature brain remains a challenge.

As we used a relatively high dose to inject and LEV is already effective in low doses in GAERS (Gower et al., 1995), we expect that relevant therapeutic concentrations are present in the blood plasma for 8 h. However, more continuous administration of the drug should be considered. This could be done by the implantation of an osmotic mini-pump that provides a continuous release and thereby more steady plasma exposure with LEV.

An interesting feature not investigated in the present GAERS study, is whether prolonged LEV treatment early in life would have modified the response to AED treatment during adulthood. In addition, also the effect of chronic LEV in adult animals could be of interest to investigate whether LEV has disease modifying properties in adult GAERS.

More recently, Krupp et al. (2000) raised the question whether treatment with AEDs during epileptogenesis induces development of drug-refractory seizures if the prevention of epileptogenesis fails. Although LEV shows potent anti-epileptogenic and anti-convulsant effects in amygdala-kindled rats, repeated treatment induces anti-convulsant tolerance and unidirectional cross-tolerance to carbamazepine administered subsequently (Zhang et al., 2003). The consequences of anti-epileptogenic treatment on the later response to AEDs in animals in which the treatment fails present a challenge that needs to be addressed in anti-epileptogenesis studies (Pitkanen and Kubova, 2004).

We hypothesized that the effect of LEV on absence seizures in GAERS could be manifested through an effect on the SNR. However, this hypothesis is based on indirect evidence from related studies. Therefore, it would be interesting to set up an experiment which determines the anatomical network and molecular mechanisms by which this effect of LEV on SNR neurons is mediated in GAERS.

Finally, future perspectives must not be limited to LEV, several other AEDs on the market should be considered. For instance valproate, lamotrigine, topiramate and zonisamide have been shown to possess interesting features potentially being anti-epileptogenic (Bolanos et al., 1998; Halonen et al., 2001a; DeLorenzo et al., 2002; Hashimoto et al., 2003). However, at present, none of these compounds has been shown to completely protect against epileptogenesis. Of course, these drugs have been selected for their ability to block seizures. It is not known whether there is a pivotal correlation between anti-seizure capability and the effect on epileptogenesis. Therefore new screening procedures, testing for effects on the processes of epileptogenesis, could reveal new classes of drugs.

Clearly, serious efforts have already been made to understand epileptogenesis and anti-epileptogenesis; however, we still have a long way to go to find the ultimate compounds and treatment strategies.

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6.2. Vagus nerve stimulation

During the current research, several aspects of VNS have been approached using animal models of epilepsy. In a chronic model of primary generalized epilepsy, namely GAERS, we investigated the effect of both acute and chronic VNS. In a chronic model of partial epilepsy (kindling in Fast rats), we investigated several therapeutic properties of VNS like the acute abortive effect of VNS on generalized convulsions, chronic effects of VNS on the development of epilepsy, cognitive effects and effects on body weight regulation. In addition, we were able to detect changes in glucose metabolism after acute and chronic VNS in healthy rats, providing a tool to further elucidate the mechanism of action (MOA) of VNS.

6.2.1 Efficacy of VNS in animal models of epilepsy

Jacob Zabara published two abstracts in Epilepsia in 1985 on the efficacy of VNS, which unleashed further research on VNS as a new therapeutic approach to treat epilepsy. He found a reduction in seizure duration and severity when stimulating the vagus nerve in dogs. One year later, Joan Lockard (1986) showed that is was feasible to implant a VNS cuff-electrode and stimulating device in a monkey model of epilepsy on a long-term basis.

At present, VNS is an efficacious broad-spectrum add-on treatment for patients with medically or surgically refractory epilepsy (Ben-Menachem, 2002). Treatment involves stimulation of the left vagus nerve via an implanted pulse generator in an attempt to reduce the frequency and severity of epileptic seizures. Controlled randomized trials using this treatment strategy have shown a 50% reduction in overall seizure frequency in approximately 30% of patients (Salinsky, 2003). In addition, VNS seems to have effects on interictal epileptiform discharges (IED). Koo (2001) observed a decrease in interictal spikes, which was later on confirmed (Kuba et al., 2002).

The benefits of VNS as a form of seizure control are numerous. Firstly, adverse effects are minimal and not additive with those frequently encountered with AEDs such as sedation and ataxia. Problems of drug toxicity, inherent to chronic medical therapy and rapidly increasing with polytherapy are avoided with VNS. Secondly, because the device is programmed to deliver continuous intermittent stimulation, there is never an issue with respect to treatment compliance. Thirdly, unlike many AEDs, the effectiveness of VNS is maintained during prolonged treatment without development of tolerance or becoming refractory to treatment. Lastly, VNS does not seem to oppose the action of additional AEDs.

Initially, animal studies on VNS preceded the use of VNS in humans, but currently, animal experiments are still ongoing trying to optimize VNS therapy and to elucidate the MOA. A chronological overview of these studies is presented in table 1. Mostly, acute VNS

studies were performed utilizing application protocols immediately before, during and/or after seizure provocation.

Table 5: Chronological overview of the efficacy of VNS in animal models of epilepsy.

Date	Researcher	Model	Animal	Result
1952	Zanchetti et al.	Strychnine	Cats	Blocking of IED and desynchronization of the cortical EEG.
1967, 1968	Stoica and Tudor	Strychnine	Cats	Decrease of IED and prevention of spreading to the contralateral side by VNS. Increase of IED by high-intensity VNS.
1990, 1991	Woodbury and Woodbury	3-MPA/ PTZ/ MES	Rats	Interruption of seizures, abortive effect.
1990	Lockard et al.	Alumina gel	Rhesus monkeys	Reduction of the number of seizures, prophylactic effect by chronic high-intensity VNS.
1992	Zabara	Strychnine/ PTZ	Dogs	Inhibition of generalized seizures by high-intensity VNS.
1993	McLachlan	Penicillin/ PTZ	Rats	Decrease of IED and seizure duration, abortive effect.
1996	Takaya et al.	MES	Rats	Dose response and prophylactic effect.
1997	Naritoku and Mikels	Kindling	Rats	Delay of kindling development.
1999	Fernandez-Guardiola et al.	Kindling	Cats	Delay of kindling development.
2001	Sunderam et al.	3-MPA	Rats	Moderate anti-seizure effect.
2002	Munana et al.	Refractory epilepsy	Dog	Cumulative efficacy of chronic VNS after long-term treatment.
2004	Dedeurwaerdere et al.	GAERS	Rats	VNS can prolong SWDs. Sub-acute VNS had no effect.
2005a	Dedeurwaerdere et al.	GAERS	Rats	High-intensity (perceptible) VNS aborts SWDs. One week of VNS does not suppress SWDs.
Submitted (a)	Dedeurwaerdere et al.	Kindling in Fast rats	Rats	Prevention of seizures and reduced ADD when VNS is applied after the kindling stimulus. No delay of kindling development and prolongation of convulsions when VNS is applied for 2 h before the kindling stimulus.

Abstracts are only included if they are not published later on in full article format. Abbreviations: IED, interictal epileptiform discharges; VNS, vagus nerve stimulation; 3-MPA, 3-mercaptoproprionate; PTZ, pentylenetetrazol; MES, maximal electroshock seizure; SWDs, spike and wave discharges; ADD, afterdischarge duration.

Zanchetti et al. (1952) were among the first to experiment with the anti-convulsive properties of VNS. They showed that interictal spikes, produced by topical application of strychnine to the cerebral cortex of the cat, were blocked by low-intensity VNS (output

current: 1-2 V, pulse duration: 0.5 ms and frequency 50 Hz). Similarly, Stoica and Tudor (1967, 1968) found that low-intensity VNS (output current: 1-4 V, pulse duration: 0.3 ms and frequency: 30 Hz) decreased the frequency of spiking activity by about 34% induced by strychnine applied topically to the coronal, anterior sigmoid, suprasylvian or medial marginal gyri of the cat. Spread to the contralateral side was also inhibited by VNS. Stoica and Tudor (1968) stated that the vagal afferent projections to these areas are diffuse and non-specific and that a different number of fibers project to each area. On the contrary, they also noted an increase in interictal spike frequency when high-intensity stimulation of the vagus nerve was performed (Stoica and Tudor, 1968). Two decades later, Woodbury and Woodbury (1990, 1991) reported that acute high-intensity stimulation at frequencies greater than 4 Hz prevented or reduced chemically (3-mercaptoproprionate, 3-MPA and pentylenetetrazole, PTZ) induced clonic convulsions in anesthetized rats and electrically (maximal electroshock seizure, MES) induced clonic and tonic clonic seizures in awake rats. This effect was attributed directly to the fraction of C fibers stimulated. It was observed that VNS shortened, but did not shut down a chemically induced seizure once it had begun. Optimal stimulation parameters were described as: output current 0.2-0.5 mA/mm² of nerve cross-section, pulse duration 0.5-1 ms and frequency 10-20 Hz. In 1992, Zabara published his work in manuscript form on the anti-convulsant effect of VNS in epileptic dogs. He found that high-intensity VNS could interrupt or terminate strychnine-induced seizures and PTZ-induced tremors in anesthetized dogs. Inhibitory effects of VNS persisted for a considerable time after termination of stimulation. It was suggested that small unmyelinated nerve fibers must be stimulated and that the optimum parameters were: output current 10-20 V (~3-20 mA), pulse duration 0.2 ms and frequency 20-30 Hz. McLachlan (1993) found that low-intensity VNS not only suppressed PTZ-induced seizures (within 3 s after seizure onset), but also reduced (by 33%) focal interictal spikes produced by penicillin in anesthetized rats. Interictal spikes could already been suppressed at 0.2 mA; however, higher currents > 0.8 mA (pulse duration: 0.5 ms and frequencies 20-50 Hz) produced a more consistent response and persisted up to 20 s after stimulation. Vagal stimulation caused no change in seizure duration in high-dose PTZ induced seizures, which was explained by a more intense ictal involvement of the brainstem reticular system. Takaya et al. (1996) found that VNS pretreatment (output current: 1 mA, pulse duration: 0.5 ms and frequency: 30 Hz continuously for 0.1 or 60 min or intermittently - 30 s/5 min on/off - for 60 min) induced a sustained anti-convulsive effect on PTZ-induced seizures in awake animals, which efficacy was dependent on the cumulative stimulus duration. Sunderam et al., (2001) reported the effect of left VNS (output current: 1.4 mA, pulse duration: 0.5 ms, frequency: 20 Hz and duty cycle: 30 s/3 min on/off) and left sciatic nerve stimulation on 3-MPA-induced seizures using a highly complicated study design. VNS only had a small anti-seizure effect, which the authors ascribed to a hemodynamically-

induced deficit in energy substrates. In a chronic model of spontaneous absence epilepsy namely GAERS, we showed a transient increase in seizure duration following acute VNS (output current: 3 V, pulse duration: 0.5 ms and frequency: 30 Hz), which disappeared in a sub-acute setting (Dedeurwaerdere et al., 2004). However, when VNS was applied at higher intensities (5-10 V) perceptible for the animals, the typical spike and wave discharges (SWDs) were shut down immediately (Dedeurwaerdere et al., 2005a). In amygdala kindled Fast rats (genetic strain of seizure prone rats), acute VNS (output current: 0.5 mA, pulse duration: 0.5 ms, frequency: 30 Hz for 60 s) showed clear anti-convulsant effects in a subset of animals when applied immediately after the kindling stimulus. However, when VNS (duty cycle: 30 s/1.1 min on/off) was applied for two hours before the kindling pulse, generalized convulsions were prolonged.

Only a few studies have assessed the effect of chronic VNS in animal models of epilepsy (Lockard et al., 1990; Munana et al., 2002; Dedeurwaerdere et al., 2005a). A possible reason for this is that long-term research on VNS in laboratory animals like rats has some practical implications. Firstly, chronic studies are laborious and time consuming, because they require long-term monitoring of the animal and subsequent analysis of all data. Secondly, there are also technical aspects, which make chronic VNS stimulation difficult to apply in small animals. To deliver chronic VNS, a pulse generator has to be implanted or VNS has to be delivered via flexible leads connected to an external stimulator. Because the Cyberonics stimulating device is rather big to implant in a rat, we chose for the latter option. We have constructed a long-term video EEG monitoring unit equipped with spring covered flexible leads (to avoid gnawing at the leads by the rat), a spring (allows up and down movements of the rat) and a communicator or swivel (allows the rat to walk around freely in the cage). The lead ends of the cuff-electrode have to be easily accessible, to connect the rat to the VNS pulse generator without difficulties. In addition, these lead ends have to be safely secured in a way that the animals cannot pull or move the leads. Therefore, we have fixated the lead ends together with the EEG electrodes with dental acrylic on the head of the rat. Finally, VNS necessitates implantation of a cuff-electrode, which is well tolerated by the animals, does not induce nerve damage and stays functional over prolonged periods. In our laboratory, such a stimulation electrode is available now (Dedeurwaerdere et al., 2005b).

A study in rhesus monkeys showed that chronic VNS was feasible and that epileptic processes are influenced (Lockard et al., 1990). However, at that time, the authors stated that safety and efficacy of the procedure were still in question. Munana et al. (2002) evaluated the use of VNS (output current: 0.25-1 mA, pulse duration: 0.5 ms, frequency: 30 Hz and on/off cycle: 30 s/ 5 min) as a treatment for refractory epilepsy in dogs. No significant difference in seizure frequency, duration or severity was detected between overall 13-week treatment and control periods. However, during the final four weeks of the treatment period, a significant

decrease in mean seizure frequency (34.4%) was found, compared with the control group. This may reflect an increase in the efficacy of VNS over time. When chronic VNS (output current: 1.5 mA, pulse duration: 0.5 ms, frequency: 30 Hz and duty cycle: 60 s/12 s on/off) was applied during one week in GAERS, the decrease in SWDs did not significantly differ from the control group (Dedeurwaerdere et al., 2005a). It can be hypothesized that a longer period of VNS or earlier intervention during life might be required to affect an already established absence epilepsy syndrome.

The effect of VNS on epileptogenesis has been investigated in the amygdala kindling model of temporal lobe epilepsy. During the kindling process seizure severity and duration gradually progress. It offers the advantage that seizures can be elicited at will and that it allows detailed study of the events associated with the epileptogenic process. In cats, it was found that VNS trains (output current: 1.2-2 mA, pulse duration: 0.5 ms and frequency: 30 Hz for 1 min) before and four times after the kindling stimulus interfered with epileptogenesis (Fernandez-Guardiola et al., 1999). These findings were confirmed in a previous abstract of Naritoku and Mikels (1997) in rats, who showed that VNS before each kindling stimulus delays the completion of kindling. However, we could not reproduce these observations using a genetic seizure prone rat strain. Kindling rate was not delayed by VNS (output current: 0.5 mA, pulse duration: 0.5 ms, frequency: 30 Hz and duty cycle: 30 s/1.1 min on/off) and during the stage-5 convulsions evidence of hyperexcitability was even noted in the VNS group (Dedeurwaerdere et al, submitted). It is conceivable that VNS cannot interfere with epileptogenesis in individuals with a strong genetic predisposition to develop epilepsy, like the Fast rats in our study. Moreover, kindling might involve different mechanisms in Fast rats compared to healthy animals. It is noted that in this study we saw that some VNS-treated rats (n= 3) showed delayed kindling rates compared to controls (n= 3) when the piriform cortex, rather than the amygdala, served as the kindling focus (S. Dedeurwaerdere, personal observations, 2004). Although, kindling rate in the piriform cortex is faster than in the amygdala (McIntyre et al., 1999), VNS rats did not display stage-5 seizures unless VNS was terminated (by lead fracture). Therefore, the origin of epileptogenesis and seizure generation could also be an influencing factor whether VNS can act on kindling development in Fast rats.

It is impossible to extract an ideal set of stimulation parameters from these studies. Most studies use frequencies between 20 and 30 Hz and a pulse duration of 0.5 ms. However, the probably most crucial parameter namely the output current is difficult to evaluate. The actual current delivered to the nerve is depending on the construction of the electrode. Different electrode types have been used and in addition, studies have been performed in anesthetized animals as well as in awake animals. In human, output current is increased till tolerability level.

Zabara (1992) observed that right or left vagal stimulation is equally effective in controlling motor seizures, but bilateral vagal stimulation produced no measurable greater effect than did unilateral stimulation. Krahl et al. (2003) confirmed that right-sided VNS was just as effective as left-sided VNS in reducing the severity of PTZ-induced seizures. They, however, speculated that stimulation of both vagus nerves may affect a larger portion of the brain than unilateral VNS. This was the case for trigeminal nerve stimulation (Fanselow et al., 2000), though was not observed by Zabara in earlier VNS studies using anesthetized dogs. Stimulation of the right or left nerve of Hering (ninth cranial nerve) can also successfully control focal seizure activity, whereas stimulation of the twelfth cranial nerve fails to suppress seizure activity (Patwardhan et al., 2002).

6.2.2 Emerging indications for VNS treatment

Vagal stimulation may affect numerous brain structures involved in the regulation of mood and cognition (Schachter, 2004). Several studies report improved alertness, behavior and mood following VNS independent of changes in seizure frequency (Elger et al., 2000; Harden et al., 2000; Harden, 2001; Kossoff and Pyzik, 2004). Accordingly, VNS has been explored as a treatment for depression (Rush et al., 2000; Sackeim et al., 2001a; Sackeim et al., 2001b; Sjogren et al., 2002). Also in animal studies, VNS has shown to be an effective antidepressant in the forced-swim test in rats (Krahl et al., 2004).

An open-label pilot study suggested a positive effect on cognition of VNS treatment for patients with Alzheimer's disease (Sjogren et al., 2002). Clark et al. (1995) hypothesized that vagal afferents affect central operations involved in the modulation of memory consolidation processes. In rats and humans, they reported that VNS was able to enhance memory storage when applied during memory consolidation, which was dependent on an inverted-U shaped function of output current (Clark et al., 1995; Clark et al., 1998; Clark et al., 1999). In contrast, chronic VNS application in a clinical setting has not been found to affect cognitive performance in patients with epilepsy since standardized tests have not identified systematic positive or negative changes in attention, motor function, short-term memory, learning and memory or executive functions following chronic VNS (Hoppe et al., 2001; Dodrill and Morris, 2001). Therapeutic agents such as AEDs, on the contrary, often exhibit mild to serious effects on cognition (Ortinski and Meador, 2004). In Fast rats, a genetic strain with learning impairment, we found that VNS stimulation was devoid of cognitive side effects in the Morris water maze, which is used to investigate spatial memory in rats. In the Alzheimer study and the studies of Clark et al. (1995, 1998, 1999) intermediate stimulation parameters were used, whereas our study and those performed in patients with epilepsy (Hoppe et al., 2001; Dodrill and Morris, 2001) used high stimulation parameters (up

to tolerability level), which are believed to have anti-epileptic effects. Therefore, different stimulation parameters may result in a different therapeutic effect.

Obesity is the most significant health problem facing westernized societies being the primary risk factor for diabetes and obstructive sleep apnea (Roslin and Kurlan, 2001). In addition, it increases the risks for heart disease, pulmonary disease, infertility, osteoarthritis, cholecystitis and several major cancers, including breast and colon cancers (Roslin and Kurlan, 2001). It is estimated that 50 to 60% of the population is obese or overweight of whom 5 to 6% are considered morbidly obese (Roslin and Kurlan, 2001). Surgical procedures for morbid obesity are becoming more common because of long-term successful results (Mason, 1992). However, optimal treatment for morbid obesity is not yet at hand (Roslin and Kurlan, 2001).

Weight reduction has been reported in patients treated with VNS (Burneo et al., 2002; Kneedy-Cayem et al., 2002). Vagal afferents play a predominant role in the regulation of food intake (Schwartz, 2000). They conduct signals from the stomach to the nucleus of the solitary tract (NST) carrying information about the size and chemical composition of a meal, which is transmitted by other specific connections to the satiety center in the hypothalamus and the collateral ventro-medial nucleus (Laskiewicz et al., 2004). A pilot-study in humans has been set up with subdiaphragmatic stimulation of the vagus nerve. This study was based on the finding in dogs that chronic bilateral VNS induced a decrease in weight, which was suggested to result from changes in the central nervous system and a secondary alteration of food intake by VNS.

In a study in GAERS, we found a significant weight reduction after two weeks of chronic VNS (Dedeurwaerdere et al., 2003). In addition, we found that VNS treatment prevented the gradual increase in body weight observed in control rats throughout the kindling process. A basic association between amygdala kindled seizures and weight gain in rats has been documented (Loscher et al., 2003; Bhatt et al., 2004). Interestingly, the amygdala has been suggested as a region where interactions between gustatory and vagal input take place (Han et al., 2003). This reduction in kindling-induced weight gain in VNS rats was associated with reduced food intake compared to control kindled rats. As in the kindling study the Fast rats only received two hours of VNS a day, it is possible that more continuous VNS would result not only in reduced weight gain, but also in reduced body weight as in GAERS. We are the first to report altered body weight in animal studies evaluating the efficacy of VNS on epilepsy likely because previous studies have primarily applied VNS acutely over very short time spans. In addition, our studies showed that in animals, weight can be regulated by chronic unilateral left VNS applied through the vagus nerve in the neck.

6.2.3 Mechanism of action of VNS

Desynchronization of the background EEG by stimulation of vagal C fibers

The idea of using VNS to suppress epileptic seizures arose from early animal experiments in which background EEG was desynchronized by VNS. As seizures result from hypersynchronous firing of a group of neurons, it was hypothesized that stimuli that produced desynchronization of the EEG may have anti-epileptic properties.

Experiments on "encéphale isolé" cats showed that stimulation of the vagus reduced the amplitude of the EEG background, produced EEG desynchronization and blocked sleep spindles during slow wave sleep (Zanchetti et al., 1952). However, these effects were not obtained in animals without spindling and the effect was similar to that of other alerting stimuli (nose rubbing, whistling). Forty years later, unilateral and bilateral VNS in cats induced, within less than 5 s, changes in the pattern and periodicity of EEG spindles, associated with depressed background rhythms or rhythmic EEG activities (Balzamo et al., 1990).

In apparent contrast, several studies observed VNS induced synchronization of the EEG. As early as 1938, Bailey and Bremer reported that VNS in the cat elicited synchronized activity on the orbital-frontal cortex EEG. Rojas (1964) showed that direct current stimulation (15-60 s) of the vagus nerve first produced EEG desynchronization with subsequent synchronization. Also Puizillout and Foutz (1974) reported that VNS produced synchronization of the EEG. McLachlan (1993) observed that background rhythms were not altered by VNS in the majority of the animals, however, in a few animals a slight increase in synchrony was noted after low-intensity and low-frequency (2-3 Hz) stimulation (McLachlan, 1993).

Stimulation of the solitary tract, which receives projections of the vagus nerve, at low frequencies (1-16 Hz) produced EEG synchronization, whereas high-frequency (> 30 Hz) stimulation results in EEG desynchronization from a sleep spindle like background also comparable to that observed with tactile or auditory stimuli (Magnes et al., 1961). Neither synchronization nor desynchronization could be elicited when the EEG background was that of arousal. Chase and colleagues (1966) performed more detailed experiments and showed a complex relation between VNS and EEG rhythmicity associated with the activation of specific nerve fiber types of the vagus. In encéphale isolé cat preparations, stimulation of the vagus at frequencies above 20 Hz and at intensities greater than 3 V produced EEG desynchronization or rather a blockage from spindle like activity. At frequencies above 70 Hz and at intensities less than 3 V, assuming that these parameters only activate myelinated fibers, vagal stimulation produced EEG synchronization. However, the changes elicited were often inconsistent and varied with the background activity. In a study in humans, both

synchronization and desynchronization of the EEG was present during long-term VNS utilizing standard stimulation parameters, which was most prominent in patients with much epileptiform activity (Koo, 2001).

The vagus nerve mainly consists of unmyelinated C fibers (65-80%) and a smaller portion myelinated A and B fibers (Rutecki, 1990). It was assumed that high-intensity and high-frequency (20-70 Hz) stimuli induce desynchronization resulting from the activation of unmyelinated C fibers, whereas, low-intensity high-frequency stimulation induces synchronization by activating myelinated A and B fibers only. However, this supposition is most likely oversimplified, because several studies contradict and the distinction between low- versus high-intensity and low- versus high-frequency stimulation is often vague.

In the study of Takaya et al. (1996), using awake and freely moving animals, no obvious VNS-induced changes in background EEG activity were observed. In addition, early studies in humans revealed that VNS induces little if any effect on EEG background rhythms. (Hammond et al., 1992a; Salinsky and Burchiel, 1993). Hence, it was hypothesized that acute desynchronization of background EEG activity is not a prominent feature of VNS when administered during physiologic wakefulness and sleep, nor does it explain the anti-convulsant effect of VNS. In these studies C fibers were probably not affected and this could be a reason why desynchronization of the EEG was not observed. Indeed, effective therapeutic stimulation parameters appear to be subthreshold for vagal C fibers in humans as there are no clinical reports of autonomic side effects (significant gastrointestinal, cardiac or respiratory effects), which would arise from C fiber stimulation (Krahl et al., 2001). These findings were supported by studies in humans who performed intraoperative nerve potential recordings (Koo et al., 2001; Evans et al., 2004). Their results showed that predominantly A and B fibers and not C fibers are activated by the stimulation output currents used in humans. Therefore, C fiber activation cannot fully explain the MOA of VNS. Moreover, Krahl et al. (2001) showed that selective destruction of capsaicin-sensitive C fibers did not affect the anti-convulsive effects of VNS on PTZ-induced seizures in rats.

Anatomical and functional projections of the vagus nerve into the cerebral hemispheres

The vagus nerve is a mixed cranial nerve that consists of ~80% afferent fibers and 20% efferent fibers parasympathically innervating heart, lungs and gastrointestinal tract (Figure 1). Mechanistic VNS research generally presumes that VNS exerts its effect through the induction of action potentials in the afferent fibers of the left vagus nerve (Zagon et al., 1999; Henry, 2002). Indeed, the anatomical diffuse projections of the vagus nerve in the cerebral hemispheres would support a broad effect for VNS on neural excitability (Rutecki, 1990) (Figure 1).

Moreover, vagal stimulation produces evoked potentials (EPs) which have been recorded from several brain regions. In the parietal association cortex, VNS evoked slow hyperpolarization in the pyramidal neurons (Zagon and Kemeny, 2000). This hyperpolarization of the membrane could reduce the excitability of these neurons. In these anesthetized rats, stimulus intensities that predominantly activated myelinated fibers (less than 200 μA) were more effective in inducing long-lasting inhibitory effects than higher stimulus intensities that activated unmyelinated vagal afferents. Furthermore, stimulation of the cervical vagus produced EPs in the cerebral cortex of several animal models (O'Brien et al., 1971; Car et al., 1975) and human (Tougas et al. 1993). In the cat thalamus, low-intensity vagal nerve stimuli facilitated cellular firing in the reticular nuclei and depressed cellular firing in the ventro-postero-medial nucleus of the thalamus (Car et al., 1975). Higher intensity vagal stimulation increased firing frequency and duration of discharges in both nuclei. EPs have also been measured in the brainstem (Bennett et al., 1988), cerebellum (Dell and Olsen, 1951; Hennemann and Rubia, 1978) and the hippocampus (Serkov and Bratus, 1970) of animals.

Functional imaging of the effect of VNS

Naritoku et al. (1995) demonstrated expression of c-*fos* (reflecting high neuronal activity) immunoreactivity in several brain regions of anesthetized rats after VNS. These brain regios included limbic structures, thalamic nuclei and brainstem noradrenergic nuclei important in the control of seizures (Naritoku et al., 1995) and supports the idea that the anti-epileptic action of VNS involves pathways that project from brainstem to forebrain.

Non-invasive mapping of anatomical sites of increased or decreased synaptic activity can be performed with functional imaging. The influence of VNS on brain activity has been demonstrated by imaging studies using positron emission tomography (PET), single photon emission computed tomography (SPECT) or functional magnetic resonance imaging (fMRI) techniques (Henry et al., 1998; Vonck et al., 2000; Narayanan et al., 2002; Sucholeiki et al., 2002).

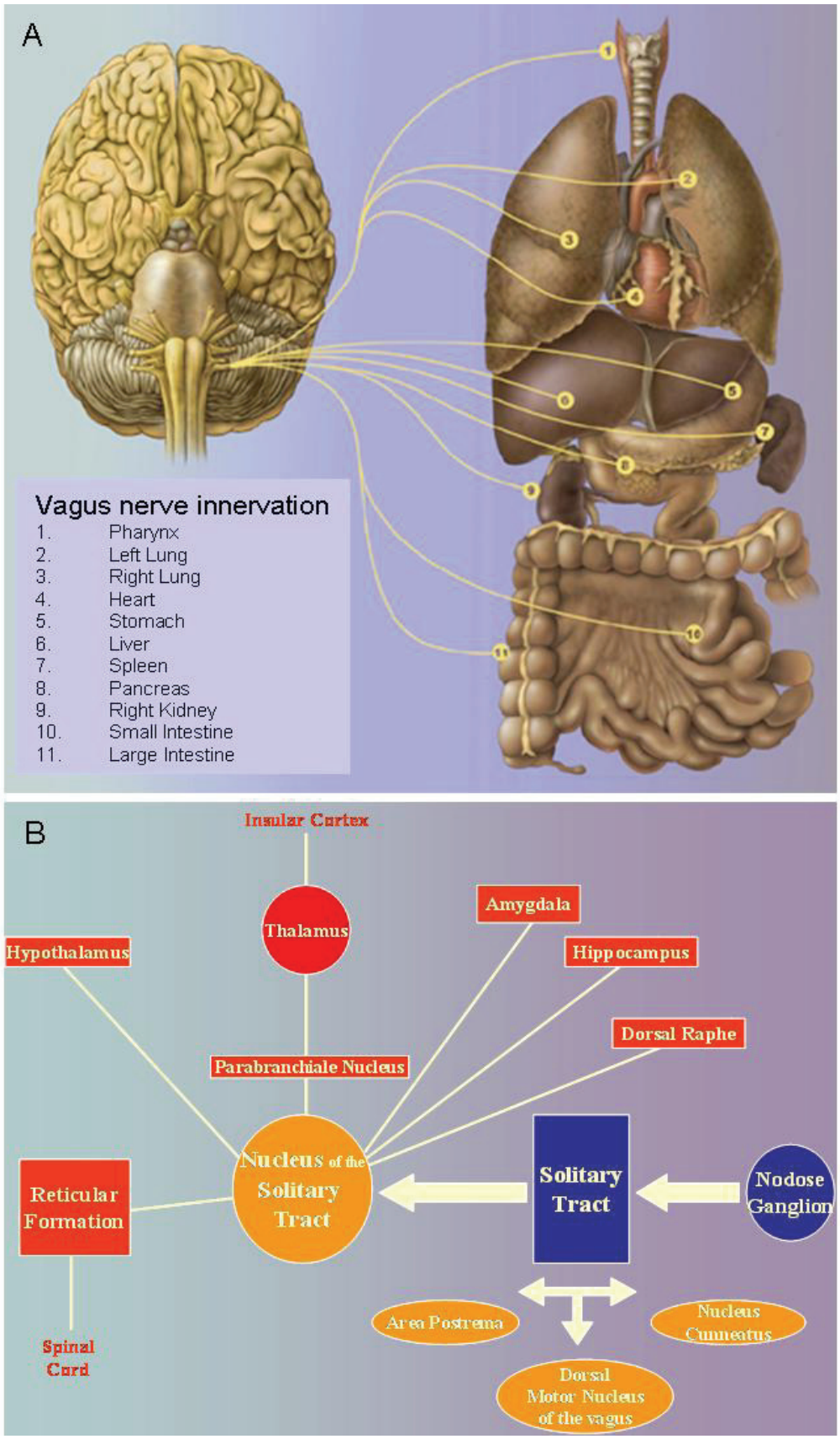

Figure 14: Efferent and afferent projections of the vagus nerve. A. The vagus nerve had efferent projections towards the pharynx, lungs, heart and gastrointestinal tract. B. Anatomical diffuse projections of the vagus nerve to cerebral hemisphere structures adapted from Rutecki et al. (1990).

These imaging studies found changes on both sides of the brain by unilateral left VNS and pointed out a key role for the thalamus and medial temporal lobe structures in the MOA of VNS. However, there is no consensus on other activated structures neither on the type of changes (inhibition or excitation). This discrepancy can be attributed to a number of confounding factors such as imaging techniques used (PET, SPECT, fMRI), tracer and contrast agents, scanning protocols, stimulation parameters, medication regimes, course of the illness and treatment response. Heterogeneity of relatively small patient samples is difficult to avoid. In addition, data gathering from healthy subjects is impossible for ethical reasons due to the invasiveness of VNS.

We found that changes in glucose metabolism due to VNS in healthy rats can be monitored using small animal PET, avoiding confounding factors as described above (Dedeurwaerdere et al., 2005c). In this animal study, results have been observed consistent with several previous imaging studies in epileptic patients (Vonck et al., 2000; Van Laere et al., 2002; Henry et al., 1998; Henry et al., 2004). During acute VNS, we found bilateral decreases in the glucose metabolism of the hippocampi (tendency in the right hippocampus), which are known to be often involved in the generation of complex partial seizures in temporal lobe epilepsy. A decreased metabolism in this region may reflect the anti-convulsant activity of acute VNS. More specifically, in patients, acute VNS can be triggered at seizure onset by a magnet to interfere with the seizure. In line with the long-term study of Henry et al. (2004), these changes in hippocampi were absent after chronic VNS. It is believed that these differences between acute and chronic VNS reflect the brains adaptation to chronic VNS. At this moment we cannot explain these changes after chronic VNS treatment observed in imaging studies. Further study will be required to determine why the acute effects of VNS on brain blood flow differ from VNS effects after months or years of stimulation. Henry (2002) suggested that intrasubject differences between acute and chronic VNS activation scans may reveal processes of adaptation to chronic VNS, and that some of them may have an anti-epileptic effect. These adaptation processes might underlie the finding that therapeutic VNS often causes gradual improved seizure control over several months of treatment (Vonck, 2003). In this case, ‘plasticity’ instead of ‘adaptation’ could be a more suitable term as 'adaptation' would in fact be expected to suppress the effect of VNS and therefore reduce the anti-convulsive effect with time

We also observed that acute VNS bilaterally increased glucose uptake in the olfactory bulbs. Evoked potential and unit activity recording techniques have revealed the existence of a vagus nerve-olfactory bulb pathway (Garcia-Diaz et al., 1984). Also in patients, VNS has been found to influence and modulate the processing of olfactory information (Kirchner et al., 2004). This neurophysiological study reported even a positive correlation between activation of the olfactory bulb and therapeutic benefit. As the olfactory bulb is on the contrary very

small in humans, it is conceivable that changes in metabolism of this structure were not measurable in human imaging studies.

Chronic VNS induced an increase in metabolism in the left medulla oblongata (Dedeurwaerdere et al., 2005c). Changes in dorsal-rostral medulla during chronic VNS have also been reported in an initial study of Henry et al. (2000). These findings correspond to the known anatomical connections and projections of the vagus nerve to the brainstem tracti and nuclei.

In addition, we found a significantly decreased left/right ratio in striatum and a tendency towards a decrease in glucose uptake in the left striatum after chronic VNS (Dedeurwaerdere et al., 2005c). This brain structure has been acknowledged to play an important role in the control of seizures (Deransart and Depaulis, 2002). Ictal hyperperfusion of the striatum was shown using single photon emission computed tomography (SPECT) in patients with temporal lobe epilepsy (Semah, 2002). The striatum receives indirect innervation from the vagus nerve via the medial reticular formation of the medulla which projects to the intralaminar nuclei of thalamus (having extremely widespread projections to cerebral cortex and the striatum) (Henry, 2002). Moreover, in a PET imaging study in humans, changes in glucose metabolism have been found in the putamen (Ko et al., 1996) and caudate nucleus (Vonck, 2003) due to VNS.

Finally, we were not able to detect previously reported changes in thalamus (Henry et al., 1998; Vonck et al., 2000; Chae et al., 2003; Henry et al., 2004) and hypothalamus (Henry et al., 1998; Henry et al., 2004) (for review see Chae et al., 2003). The negative findings in these regions therefore do not exclude the presence of smaller or more localized changes. Further study will require higher-resolution acquisition and more advanced analysis. Imaging studies in patients of our group showed a decreased tracer uptake in the left thalamus during chronic VNS (Vonck et al., 2000; Van Laere et al., 2002; Vonck, 2003). This may demonstrate that VNS induces a chronic inhibitory state in this key structure for seizure spread, which is manifested by a decrease in blood flow. In line with this, it has been quoted that VNS could result in hemodynamically-induced hypoperfusion creating a relative deficit of energetic substrates (Sunderam et al., 2001).

Changes in neurotransmission induced by VNS

Naritoku et al. (1992) found that chronic VNS prolongs the cervicomedullary to thalamocortical potential interval during somatosensory evoked potential studies. It was concluded that chronic VNS does alter neuronal networks outside of the brain stem vagus system and that alteration of forebrain neurotransmission might be a primary mechanism of VNS.

Woodbury and Woodbury hypothesized that VNS appears to act via release of large quantities of the inhibitory mediators GABA and glycine throughout large volumes of the brain, because it counteracts PTZ, 3-MPA (decreases efficacy of all inhibitory synapses) and strychnine induced seizures (Woodbury and Woodbury, 1991). In addition, GABA-mediated inhibition is implicated in the anti-epileptic effect of NST stimulation in rats, which is the principal target of VNS (Walker et al., 1999).

Despite these experimental observations, a direct or indirect role of GABA in the anti-epileptic effect of VNS in humans remains to be demonstrated. In humans, the effect of VNS has been examined on the concentration of amino acids and neurotransmitters in cerebrospinal fluid (CSF) samples and no clear effect on GABA concentrations was determined (Hammond et al., 1992b; Ben-Menachem et al., 1995). Nevertheless, significant increases were seen in homovanillic acid (metabolite of dopamine), 5-hydroxyindoleacetic acid (metabolite of serotonin) and ethanolamine levels. Decreases were found in the level of aspartate. Although serotoninergic as well as dopaminergic systems have been found to have anti-convulsant effects in animal and human studies of various types of epilepsies, it remains to be clarified whether these findings are epiphenomena or findings directly related to VNS (Hammond et al., 1992b).

The integrity of an additional neurotransmitter system, namely norepinephrine released from the locus coeruleus, appears to play an important role in seizure suppression (Krahl et al., 1998). VNS results in a significant increase in the discharge rate of locus coeruleus neurons in rats (Groves et al., 2005). Bilateral lesions of the locus coeruleus in rats prevented the seizure-suppressing effects of VNS (Krahl et al., 1998). The authors claimed that lesioning the locus coeruleus blocks the anti-convulsant effects of VNS by preventing VNS-induced norepinephrine release either globally or in some specific brain sites. VNS also results in a long-lasting (greater than 80 min) increase in norepinephrine efflux in the basolateral amygdala (Hassert et al., 2004). Noradrenergic projections to the amygdala arise from the locus coeruleus, which is the largest population of noradrenergic neurons in the brain and which receives projections from the NST, thus could be modulated by the vagus (Van Bockstaele et al., 1999). It is hypothesized that part of the mechanism of kindling may be a decrease in the effects of norepinephrine (Loscher et al., 2003). Interestingly, we found that VNS could suppress generalized convulsions evoked by amygdala kindling in some animals (Dedeurwaerdere et al., submitted). Moreover, electrical stimulation of the locus coeruleus suppresses epileptiform activity produced by stimulation of the amygdala (Jimenez-Rivera et al., 1987). Taken together, these data suggest that activation of the locus coeruleus by VNS might be a significant factor for the attenuation of seizures.

The decrease of norepinephrine in various forebrain regions (including the stimulated amygdala) due to kindling could, besides the induction of seizures, also be a possible reason

for kindling-induced weight gain (Loscher et al., 2003). Drugs to treat obesity include noradrenergic drugs that reduce food intake (Bray, 2000). This could be of possible relevance to the finding that VNS reduced kindling-induced weight gain in Fast rats, which was associated with a reduced food intake compared to control kindled rats (Dedeurwaerdere et al., submitted). In conclusion, an additional role of VNS-induced changes in norepinephrine in the amygdala could include the regulation body weight and decreased food intake.

The effects of chronic VNS on satiety and food intake could suggest an additional anti-convulsant MOA of VNS by caloric restriction. Diets have been used for the treatment of intractable childhood epilepsy since the 1920s and are re-emerging as a treatment option (Kossoff, 2004), although the MOA of these diets may be related to induced ketosis.

Acute and chronic VNS

Most likely there is not one MOA of VNS, but rather a summation of several physiological phenomena attributing to the overall effect of VNS. This is also apparent in the differences between acute and chronic VNS. Acute and chronic VNS may act via two distinct mechanisms of action which is supported by imaging studies in humans (Vonck et al., 2000; Van Laere et al., 2002).

Acute VNS could be mediated by a possible mechanism of non-specific arousal by stimulation of somatic sensory pathways (Rutecki, 1990; McLachlan, 1993; Fanselow et al., 2000). McLachlan et al. (1993) observed that similar reduction in spike frequency by VNS was obtained by thermal stimulation of the tail. He hypothesized that VNS influences epileptiform activity by a nonspecific mechanism mediated through the reticular activating system. When acute VNS was applied during absence seizures in GAERS at high intensity, noticeable for the animals, SWDs were stopped (Dedeurwaerdere et al. 2005a). Indeed absence seizures are aborted by unexpected sensory stimuli (e.g. noise, touching the animal) in GAERS (Danober et al., 1998). On the other hand, when VNS was applied at lower intensity, not perceptible by the animal, seizures were not interrupted and even prolonged (Dedeurwaerdere et al., 2004, 2005a). We also found that VNS reduced amygdala evoked seizures (Dedeurwaerdere et al. submitted). However, it has been observed that kindling-induced seizures can be stopped by sensory stimulation (K. Gilby, pers. com.). Moreover, kindled seizures can be blocked by an antecedent footshock (Pinel et al., 1973). PTZ-induced seizures can also be reduced by trigeminal and Hering nerve stimulation (Fanselow et al., 2000; Patwardhan et al., 2002). In humans, it has been demonstrated that sensory stimulation can suppress focal spikes and absence seizures (Ricci et al., 1972; Rajna and Lona, 1989). In patients with VNS, output current is typically programmed higher in the magnet mode to obtain this arousal effect. Acute VNS could likely be mediated by nonspecific arousal, although other or additional mechanisms of action cannot be excluded.

Chronic VNS is administered in an intermittent way and it appears that seizures occurring during the VNS off-time are also affected. This intermittent way of stimulation is insufficient to explain the reduction of seizures on the basis of abortive effects alone and suggests a true preventative or so-called anti-epileptic effect of VNS (Vonck, 2003). Processes that mediate VNS-induced sustained changes are unknown, but the persistence of the anti-convulsant effects suggests that VNS induces long-term changes in neuronal activity (Takaya et al., 1996). Naritoku et al. (1995) demonstrated expression of c-*fos* immunoreactivity in several brain regions important in epileptogenesis by VNS. C-*fos* is a protein that signals transcription of other genes. Therefore, the anti-epileptic effect of VNS may be mediated through transcriptional events resulting into intermediate or long-term changes. It is reasonable that chronic VNS induces permanent changes in network connections as the efficacy of VNS generally increases after long-term treatment.

In relation to the improving long-term effect of VNS it has been reasoned out that if epileptic fits can induce more epileptic fits as is the case in kindling, then reducing the number of epileptic events (and perhaps also subclinical ones) by VNS could reduce the risk for more epileptic events and thus 'cure' the epilepsy. This mechanism could be seen as the reverse of kindling. Firstly, Gowers' (1881) famous quotation that "seizures beget seizures" is still under debate (Theodore and Wasterlain, 1999; McIntosh and Berkovic, 2005). Indeed, experimental work in animals provided support that even single seizures are harmful (Bengzon et al., 1997), which may initiate or facilitate a cascade of events leading to an epileptogenic lesion (Briellmann et al., 2005). However, in patients inconsistent data exist. Moreover, human studies suggest that epilepsy is usually not a progressive disorder although some epilepsy syndromes (e.g. progressive myoclonic epilepsies, epileptic encephalopathies) do have a progressive intractable course (Berg and Shinnar, 1997). Anyway, it is probably not by preventing the seizures themselves that epilepsy could be cured, but rather by treating the underlying cause of the seizures (Berg and Shinnar, 1997). Also our own study in young GAERS may be relevant to this discussion. Although GAERS displayed fewer seizures during development of the absence epilepsy because of the chronic LEV treatment, they all developed a similar epilepsy profile as the control group after terminating the treatment (Dedeurwaerdere et al., 2005d). Nevertheless, the hypothesis that VNS unkindles epilepsy has been tested by Dasheiff et al., (2001). They hypothesized that the longer and more frequent a person has seizures, the longer it will take to unkindle them. Therefore, they have accessed the database of two large clinical trials and multiple types of analyses could not support their hypothesis. Hence they stated that VNS does not unkindle seizures. Moreover, responders to VNS treatment do have seizure relapse after battery failure. Hence, at this moment it does not seem that VNS is curing epilepsy.

6.2.4 Conclusion

Left-sided VNS is an effective treatment for patients with complex partial seizures with or without secondary generalization who show insufficient response to anti-convulsant drugs and are unsuited for neurosurgical treatment. In addition it is a well-tolerated and safe form of neurostimulation. Indeed, we found that VNS does not induce cognitive side effects in Fast rats.

Remarkably, VNS could as well prolong as shorten SWDs in GAERS depending on the output current used. This phenomenon was also observed on amygdala kindling induced seizures in Fast rats. In this study, VNS application before the induction of a seizure prolonged seizures while VNS applied after the kindling pulse could prevent seizures. We should also note that the anti-epileptic effect of VNS on amygdala kindled seizures was only present in a small subgroup of animals. Briefly, VNS can induce both synchronization and desynchronization and can result in both excitation and inhibition as supported by our studies in a model of generalized primary epilepsy (GAERS) and a model of partial epilepsy (amygdala kindling).

Despite several discrepancies in the VNS literature, we can state that VNS activates and inhibits multiple brain regions thereby affecting several neurotransmitter systems. More specifically, in our micro-PET study we found decreased metabolism in the left hippocampus and left striatum and increases in glucose metabolism in olfactory bulbs and left medulla oblongata.

Most likely there is not one MOA of VNS, but rather a summation of several physiological phenomena attributing to the overall effect of VNS. The major MOAs underlying the desired anti-seizure actions of VNS may be mediated by its diffuse projections in the cerebral hemispheres inducing i) a transient decrease in synaptic activities in the amygdala, hippocampus and other components of the limbic system, ii) an intermittent increase in release of norepinephrine in the cerebrum or iii) a change in synaptic activities in the thalamus and thalamocortical projection pathways bilaterally, leading to increased arousal and possibly to decreased synchrony of synaptic activities between and within cortical regions. Perhaps, these separate mechanisms of VNS can be fine tuned by combining specific stimulation parameters and stimulation protocols resulting in an optimal outcome.

There is clear evidence that acute and chronic VNS differ in MOA. Whereas the anti-convulsive effect of acute VNS could be due to an arousal effect, results from different studies in humans and animals are increasingly supporting the idea that the anti-epileptic effect of chronic VNS is based on long-term modulatory changes in synaptic transmission within certain neuronal network. Hence, it takes several months for these changes to be fully installed and expressed.

Besides its anti-epileptic effect, it is not surprising that VNS, which has numerous projections to the brain, could influence several other functions like mood, memory, feeding behavior. In our studies, effect on body weight induced by VNS was indicated in two animal models of epilepsy namely GAERS and amygdala kindled Fast rats.

6.2.5 Future perspectives

Vagus nerve stimulation has been used since 1988 and at present almost 30 000 patients are being treated with VNS worldwide. Experience and knowledge about VNS is rapidly increasing, however several questions remain unclear. VNS is used in generalized and partial epilepsy (Ben-Menachem, 2002), but still responder groups have not been clearly identified. Currently 30% of patients treated with VNS will not benefit from VNS. A better understanding of VNS could improve seizure outcome by identifying specific epilepsy syndromes or types of epilepsy that respond well to VNS on the one hand or by optimizing stimulation parameters on the other hand. Future animal research is therefore crucial.

VNS efficacy in animals has primarily been assessed in acute models utilizing stimulation protocols in close relation to the time of seizure onset. These studies have pointed out a series of stimulation parameters, although there is no agreement whether high- or low-stimulation parameters should be used and which fiber groups are affected by such stimulation. Unfortunately, an adapted electrode including the measuring capability of action potentials of the different nerve fibers induced by VNS, is not yet available for the vagus nerve of rats and previous attempts were faced with lots of difficulties. However, the capability of the VNS electrode to selectively elicit different fibers can be first tested in a setup using anesthetized animals, avoiding the practical difficulties of using freely moving animals. After distinguishing sets of stimulation parameters (output current, pulse duration, frequency) that are activating different fibers, their anti-epileptic properties can be tested in a chronic model of epilepsy, like the kindling model in which seizures can be induced at will.

The efficacy of chronic VNS (several weeks of stimulation) should be investigated in different animal models of epilepsy that are closely imitating human epilepsy such as GAERS, kindling model, SE model, concussion model and stroke models. This may provide clues on the identification of responder groups. However, responders to VNS therapy are perhaps not associated with a specific type of epilepsy. In a model of temporal lobe epilepsy, VNS benefited only a subpopulation of the amygdala kindled rats while other rats appeared relatively unaffected. Further research directed towards identification of essential criteria that leads to success for VNS application is warranted. Maybe specific genes can be identified which correlate with the degree of benefit from VNS.

Presently, there are only a few guesses of which neurotransmitters contribute to the action of VNS. Further research should determine which neurotransmitters (e.g. GABA,

glutamate, norepinephrine, serotonin, dopamine) are involved in the acute and chronic effects of VNS. This could be realized by means of microdialysis investigating changes in neurotransmitters in several brain structures. Also small animal PET could be performed using specific tracers during different stimulation protocols. As the resolution of small animal PET is limited, several structures like the nucleus coeruleus cannot be visualized. Therefore, refining research using autoradiography and fMRI can be performed to investigate inhibition and excitation of anatomically small regions.

Besides its anti-epileptic effect, VNS could also influence several other functions like mood, memory, feeding behavior. However, it is conceivable that different stimulation parameters can result in a different effect. Hence, stimulation parameters probably must be optimized for each target group.

Clearly, the complexities of VNS treatment should be further investigated in order to optimize treatment in patients with refractory epilepsy.

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Summary

Summary

Epilepsy is the most common serious brain disorder affecting 0.5-1% of the general population. This neurological disorder consists of recurrent seizures, resulting from excessive, uncontrolled electrical activity in the brain. Despite the pharmacological development of new treatments, still one third of the epilepsy patients does not respond sufficiently to anti-epileptic drugs (AED) and are called refractory patients. Hence, there is a constant impetus to search for other treatment strategies like epilepsy surgery, vagus nerve stimulation and deep brain stimulation. Besides the ongoing research on the efficacy of anti-epileptic treatments in suppressing seizures (anti-seizure effect), we want to seek for therapies that can lead to plastic changes in the epileptic network and in this way have a modulating effect. The impact of therapies which may slow down processes underlying epilepsy and which might prevent or even cure epilepsy cannot be overlooked.

Neuropharmacological therapy with levetiracetam (LEV) and vagus nerve stimulation (VNS) are two novel treatments for refractory epilepsy. Acute application of both treatment options can be very effective. LEV can act rapidly on seizures in both animals and humans. In addition, preclinical studies suggest that LEV may have anti-epileptogenic and neuroprotective effects, with the potential to slow or arrest disease progression. VNS as well can have an immediate effect on seizures in animals and patients with in addition a cumulative effect after prolonged treatment. These treatments can be considered as neuromodulatory since there is evidence that changes in central nervous system function or organization are induced by influencing and initiating neurophysiological signals.

Studies in man are hampered by the heterogeneity of patient populations (age, course of the epilepsy, type of epilepsy, AED regime and genetic background) and the difficulty to study therapy-related effects in a systematic way. Therefore, investigation was performed utilizing two models mimicking epilepsy in humans. They are both chronic models with seizures evolving from true, genetically-driven epileptogenesis. Genetic absence epilepsy rats from Strasbourg (GAERS) have inborn absence epilepsy and Fast rats have a genetically determined sensitivity for electrical amygdala kindling, which is an excellent model of temporal lobe epilepsy.

Anti-epileptogenic effects of LEV in addition to anti-epileptic effects have been reported in the rat amygdala kindling model and the spontaneously epileptic rat (SER), a model of primary generalized epilepsy characterized by spontaneous tonic convulsions and absence seizures. Administration of LEV strongly suppresses the occurrence of absence seizures in GAERS. In a pilot study in GAERS, the robust anti-seizure effect of LEV was confirmed and a trend towards an anti-epileptogenic effect was found. This encouraged us to further investigate the neuromodulatory properties of LEV in GAERS; it was felt that

investigating the effect of chronic LEV treatment in young GAERS could provide new insights and strategies for the treatment of epilepsy. During the present research, we have investigated the effect of LEV on the age-related development of spike and wave discharges (SWDs) in GAERS by chronic administration of LEV (postnatal day (PN) 23-PN60) starting before the age of occurrence of SWDs. We found that chronic LEV administration induced a reduction in epileptiform events in young GAERS. This effect persisted to some extent after treatment cessation (PN61-PN64), which might indicate a slowing down of epileptogenic processes. However, this effect was only temporary, because at the age of four months all animals revealed a similar expression of epileptiform discharges. Further studies should determine the optimal time window to interfere more permanently with epileptogenesis in GAERS.

VNS has been used since 1988 and at present approximately 30 000 patients are being treated with VNS worldwide. Experience and knowledge about VNS is rapidly increasing, however several questions remain unclear. VNS is used in generalized and partial epilepsy, although responder groups have not been clearly identified. Currently 30% of patients treated with VNS will not benefit from VNS. VNS is believed to induce its effect by affecting a large number of intracerebral structures through stimulation of the vagal fibers in the neck. The precise mechanism of action by which VNS exerts its anti-epileptic effect has not been elucidated yet. Understanding VNS could improve seizure outcome by identifying specific epilepsy syndromes or types of epilepsy that respond well to VNS or by optimizing stimulation parameters.

Initial animal studies with VNS showed promising results in reducing both ictal and interictal EEG abnormalities. These findings laid the foundation for further development of VNS as a treatment for human epilepsy. However, VNS efficacy in animals has primarily been assessed in acute models (3-mercaptopropionate, pentylenetetrazole, maximal electroshock, penicillin or strychnine application) utilizing application protocols immediately before and/or after seizure provocation. Only a few studies have evaluated the effect of VNS in chronic animal models of epilepsy. It is clear that additional research is needed using chronic animal models and using both acute and chronic VNS protocols. Moreover, such fundamental data for idiopathic epilepsy are presently completely lacking. Information about the potential efficacy of VNS in GAERS, a validated animal model of absence epilepsy, could help to clarify the general principles that underlie VNS, although extensive therapeutic use of VNS in absence epilepsy is unlikely. In this chronic model of spontaneous absence epilepsy, we observed a transient increase in seizure duration following acute VNS, which disappeared in a sub-acute setting. However, when VNS was applied at higher intensities perceptible for the animals, the typical SWDs were shut down immediately. When chronic VNS was applied during one week in GAERS, the decrease in SWDs did not significantly differ from the

control group. It can be hypothesized that a longer period of VNS or earlier intervention during life might be required to affect an already established and genetically driven epilepsy syndrome. In a genetic seizure-prone kindling-strain, epileptogenesis was not prevented by two hours of daily VNS. Again both excitatory and inhibitory effects of VNS were observed. Seizure profiles in these fully kindled Fast rats were worsened when VNS was applied before the kindling pulse, whereas VNS applied immediately after the kindling pulse could completely prevent stage-5 seizures in a subset of animals. In addition, VNS stimulation appeared to be devoid of significant cognitive side effects in the Morris water maze. Finally, VNS reduced body weight after two weeks of treatment in GAERS and prevented weight gain associated with the kindling process in Fast rats presumably via the observed reduction in food intake. Clearly, the complexities of VNS treatment should be further investigated in order to optimize treatment in patients with refractory epilepsy.

Several functional imaging studies have been conducted to investigate the activation or inhibition of brain structures by VNS. These studies found changes on both sides of the brain by unilateral left VNS and pointed out a key role for the thalamus and medial temporal lobe structures in the MOA of VNS. However, there is no consensus on the type of changes (inhibition or excitation) neither on other potentially activated structures. This discrepancy can be attributed to a number of confounding factors such as the differences between the imaging techniques used (PET, SPECT, fMRI), the contrast agents, scanning protocols, stimulation parameters, medication regimes, course of the illness and treatment response. Heterogeneity of the patient samples is difficult to avoid. In addition, data gathering from healthy subjects is impossible for ethical reasons as VNS is a relatively invasive procedure. Therefore, we explored whether it is feasible to investigate the effect of acute and chronic effect of VNS on brain glucose metabolism in rats using micro-PET. We showed that acute and chronic VNS induced changes in glucose metabolism in regions important for seizure control such as hippocampus and striatum. Our pilot study demonstrated that small animal PET is a useful and promising technique for imaging cerebral activation in long-term studies in rats.

Samenvatting

Samenvatting

Epilepsie is een ernstige neurologische aandoening die voorkomt bij 0,5 tot 1% van de bevolking en die gekenmerkt wordt door het herhaald en spontaan optreden van epileptische aanvallen. Een epileptische aanval bestaat uit een paroxysmale gedrags- en/of bewustzijnsverandering ten gevolge van een excessieve neuronale ontlading ter hoogte van een deel of de gehele hersenschors. Ondanks de farmacologische ontwikkeling van nieuwe anti-epileptica, reageert een derde van de patiënten onvoldoende op medicatie (refractaire patiënten). Er is dus een continue zoektocht naar alternatieve behandelingsmodaliteiten zoals epilepsie chirurgie, nervus vagus stimulatie en diepe hersenstimulatie. Naast het bestaande onderzoek naar de doeltreffendheid van behandelingen om aanvallen te onderdrukken, willen we zoeken naar therapieën die plastische veranderingen in het epileptische netwerk kunnen aanbrengen en hierbij een modulerend effect hebben. De impact van therapieën, die epileptogene processen zouden kunnen vertragen, voorkomen en eventueel zelfs epilepsie zouden kunnen genezen, is niet te onderschatten.

Twee nieuwe behandelingen voor refractaire epilepsie zijn het anti-epilepticum levetiracetam (LEV) en nervus vagus stimulatie (NVS). LEV onderdrukt snel aanvallen zowel in dierstudies als in patiënten. Daarenboven suggereren preklinische studies dat LEV anti-epileptogene en neuroprotective eigenschappen bezit. Dit houdt in dat LEV de progressie van epilepsie zou kunnen vertragen of zelfs de ziekte zou kunnen stoppen. Ook NVS kan een onmiddellijk effect hebben op aanvallen in mens en dier met bovendien een verhoogde doeltreffendheid op langere termijn. Deze behandelingsmodaliteiten kunnen worden beschouwd als neuromodulatorisch omdat er evidentie is dat ze veranderingen teweeg brengen in de werking en organisatie van het centrale zenuwstelsel door neurofysiologische signalen te beïnvloeden en te initiëren.

Studies bij de mens worden belemmerd door de heterogeniteit van de patiënten populaties (leeftijd, duur van de epilepsie, type van epilepsie, medicatie en genetische achtergrond) en de moeilijkheid om therapie gerelateerde effecten op een systematische manier te onderzoeken. Daarom werd voor het huidige onderzoek gebruik gemaakt van twee diermodellen die dicht aansluiten bij de pathofysiologie van de mens. Het zijn beide chronische modellen met aanvallen die resulteren uit een ware, genetisch gedreven epileptogenesis. De “genetic absence epilepsy rats from Strasbourg” (GAERS) hebben een overerfbare vorm van absence-epilepsie en de “Fast rat strain” heeft een genetisch bepaalde predispositie voor het ontwikkelen van epilepsie door kindling van de amygdala, een uitstekend model voor temporale kwabepilepsie.

Naast anti-epileptische effecten van LEV zijn ook anti-epileptogene eigenschappen geobserveerd in het amygdala kindling model en in de “spontaneously epileptic rat” (SER),

een model voor primair gegeneraliseerde epilepsie gekarakteriseerd door spontane, tonische convulsies en absences. Toediening van LEV onderdrukt krachtig het voorkomen van absences in GAERS. In een pilootstudie in GAERS, werd het robuuste anti-epileptische effect van LEV bevestigd en werd tevens een aanwijzing voor een anti-epileptogeen effect gevonden. Dit moedigde ons aan om de neuromodulatorische eigenschappen van LEV in GAERS verder te onderzoeken. Tijdens het huidige onderzoek werd het effect van LEV onderzocht op de leeftijdsafhankelijke ontwikkeling van "spike and wave discharges" (SWDs) in GAERS. Chronische administratie van LEV werd gestart vóór de leeftijd waarop SWDs verschijnen op het corticale EEG. Dit onderzoek toonde aan dat chronische LEV administratie epileptiforme hersenactiviteit in jonge GAERS onderdrukt. Daarenboven was dit anti-epileptische effect nog steeds aanwezig na onderbreking van de behandeling (PN61-PN64), wat op een remming van epileptogene processen zou kunnen wijzen. Dit effect was slechts tijdelijk, want op de leeftijd van vier maanden (volwassenheid) vertoonden zowel de controle als de voordien behandelde dieren SWDs op het EEG, die niet verschilden in aantal of duur. Verdere studies zijn vereist om na te gaan of een optimaal tijdvenster kan bepaald worden waarbij een meer permanent effect op epileptogenesis in GAERS bekomen wordt.

NVS wordt toegepast sinds 1988 en momenteel worden ongeveer 30.000 epilepsiepatiënten over de hele wereld behandeld met NVS. De ervaring en de kennis over NVS groeit snel, maar verscheidene vragen blijven onbeantwoord. NVS wordt gebruikt in gegeneraliseerde en partiële epilepsie, hoewel "responders" niet duidelijk zijn geïdentificeerd. Momenteel heeft dertig procent van de patiënten behandeld met NVS geen baat bij de therapie. Het precieze werkingsmechanisme van NVS is nog niet volledig ontrafeld. Een beter begrip van het werkingsmechanisme van NVS kan de efficiëntie van de behandeling verbeteren door identificatie van specifieke epilepsiesyndromen of vormen van epilepsie die goed reageren op NVS of door optimalisatie van stimulatieparameters. De aanvankelijke dierstudies met NVS toonden veelbelovende resultaten met vermindering van zowel ictale als interictale abnormaliteiten op het EEG. Deze bevindingen legden de fundamenten voor de verdere ontwikkeling van NVS als behandeling voor epilepsie bij de mens. Nochtans is de doeltreffendheid van NVS in dieren hoofdzakelijk beoordeeld in acute modellen (3-mercaptopropionate, pentylenetetrazol, maximale elektroshock aanvalsmodel en applicatie van penicilline of strychnine), waarbij stimulatieprotocollen gebruikt werden onmiddellijk vóór en/of na de uitlokking van aanvallen. Slechts een paar studies hebben het effect van NVS in chronische diermodellen voor epilepsie geëvalueerd. Het is duidelijk dat bijkomend proefdieronderzoek, gebruikmakend van chronische modellen en zowel acute als chronische protocollen, noodzakelijk is. Voorts ontbreken fundamentele gegevens over de doeltreffendheid van NVS bij idiopathische epilepsie. Onderzoek naar de efficaciteit van NVS in GAERS, een model voor idiopathische absence epilepsie, kan helpen om de algemene

principes te verduidelijken die aan de grondslag liggen van NVS. In dit model namen wij een tijdelijke verlenging van de aanvalsduur na acute NVS waar. Wanneer echter met een hogere, voor de dieren voelbare intensiteit gestimuleerd werd, konden de typische SWDs onmiddellijk worden onderbroken. Na één week van chronische NVS waren de aanvallen niet beduidend lager dan in de controlegroep. Men kan de hypothese vooropstellen dat een langere periode van NVS of vroegere interventie tijdens het leven vereist is om een gevestigd en genetisch gedreven epilepsiesyndroom te beïnvloeden. In Fast ratten, met een genetische vatbaarheid voor amygdala kindling, werd epileptogenesis niet verhinderd door dagelijks twee uren NVS toe te dienen voor de kindling stimulus. Opnieuw werden zowel excitatorische als inhibitorische effecten van NVS waargenomen in dit model. Aanvalsprofielen bij deze volledig gekindelde ratten werden erger wanneer NVS *voor* de kindling puls werd gegeven, terwijl NVS onmiddellijk *na* de kindling puls gegeneraliseerde convulsies in een subpopulatie van de NVS-groep volledig kon verhinderen. Bovendien scheen NVS geen significante cognitieve bijwerkingen te hebben in de "Morris water maze" in deze Fast ratten. Tot slot verminderde NVS het lichaamsgewicht in GAERS na twee weken van behandeling. Daarenboven werd gewichtsaanwinst, geassocieerd met het kindling proces, verhinderd bij de Fast ratten, wat gekoppeld was aan een verlaagde voedselopname. Het is duidelijk dat de complexiteit van NVS verder moet worden onderzocht om de behandeling van patiënten met refractaire epilepsie te optimaliseren.

Verscheidene functionele beeldvormingstudies zijn uitgevoerd om de activering of de inhibitie van hersenenstructuren te onderzoeken door NVS. Deze studies vonden veranderingen aan beide zijden van de hersenen door unilaterale linker NVS en wezen op een belangrijke rol voor de thalamus en de temporale kwabstructuren in het werkingsmechanisme van NVS. Er is echter geen consensus betreffende de aard van de veranderingen (activatie of inhibitie) noch over andere hersenstructuren die eventueel aangesproken worden door NVS. Deze discrepantie kan aan een aantal factoren worden toegeschreven zoals verschillen tussen de gebruikte beeldvormingtechnieken (PET, SPECT, fMRI), de contrastagenten, de scanningprotocollen, de stimulatieparameters, de medicatie, de duur van de ziekte en respons op de behandeling. Heterogeniteit van de patiëntensamples is moeilijk te vermijden. Bovendien zijn gegevens afkomstig van gezonde individuen onmogelijk te verzamelen omwille van ethische redenen aangezien NVS een vrij invasieve procedure is. Daarom onderzochten wij of het haalbaar is om het effect van acute en chronische NVS op het glucosemetabolisme van de hersenen van de rat na te gaan met micro-PET. Wij konden aantonen dat acute en chronische NVS veranderingen veroorzaakten in het glucosemetabolisme in gebieden belangrijk voor aanvalscontrole zoals hippocampus en striatum. Onze pilootstudie toonde aan dat "small animal PET" een nuttige en veelbelovende techniek is voor de weergave van hersenactivering in lange termijn studies bij de rat.

Résumé

Résumé

L'épilepsie est une maladie grave qui se manifeste chez 0,5 à 1 % de la population et qui se caractérise par l'apparition répétée et spontanée de crises épileptiques. Une crise est associée à un changement de l'état de conscience et/ou du comportement dû à une décharge neuronale excessive dans une ou plusieurs structures corticales. Malgré le développement pharmaceutique de nouveaux anti-épileptiques, un tiers des patients réagit insuffisamment aux médicaments (épilepsie réfractaire). Il en résulte une recherche continuelle de nouveaux traitements comme la chirurgie de l'épilepsie (résection de la zone épileptogène), la stimulation du nerf vague et la stimulation cérébrale profonde. En plus de la recherche existante sur l'efficacité des traitements suppressifs, nous voulons explorer les thérapies capables non seulement d'entraîner des modifications plastiques dans le réseau épileptique mais également de présenter simultanément un effet modulateur. L'impact des thérapies qui pourraient ralentir les processus épileptogènes ne doit pas être sous-estimé.

L'anti-épileptique levetiracetam (LEV) et la stimulation du nerf vague (SNV) constituent deux nouveaux traitements de l'épilepsie réfractaire. Le LEV supprime rapidement les crises aussi bien dans les études animales que chez les patients. En plus, les études précliniques suggèrent que le LEV possède des propriétés anti-épileptogènes et neuroprotectrices, ayant la possibilité de ralentir ou même d'arrêter la progression de la maladie. La SNV peut aussi avoir un effet immédiat sur les crises chez l'homme et l'animal avec en plus une efficacité élevée à long terme. Ces modalités de traitements peuvent être considérées comme neuromodulatrices puisqu'il est évident qu'elles provoquent des changements dans le fonctionnement et l'organisation du système nerveux central en influençant et en initiant des signaux neurophysiologiques.

Les études chez l'homme sont difficiles en raison de l'hétérogénéité des populations de patients (âge, durée de l'épilepsie, type d'épilepsie, médicaments et terrain génétique) et à cause de la difficulté d'explorer les effets reliés à la thérapie d'une manière systématique. C'est pour cette raison que nous avons décidé de nous concentrer sur deux modèles animaux qui se rapprochent fortement de la pathophysiologie humaine. Ce sont des modèles chroniques avec des crises résultant d'une épileptogenèse liée à des modifications génétiques. Les « genetic absence epilepsy rats from Strasbourg (GAERS) » présentent une forme d'épilepsie de type absence héréditaire et le « Fast rat strain » est caractérisé par une prédisposition génétique pour le développement de l'épilepsie par « kindling » de l'amygdale, un modèle validé de l'épilepsie du lobe temporal.

En plus des effets anti-épileptiques du LEV, des propriétés anti-épileptogènes furent observées dans le modèle de « kindling » de l'amygdale et chez le « rat épileptique spontané (RES) », un modèle d'épilepsie généralisée caractérisé par des convulsions toniques spontanées ainsi que des absences. L'administration du LEV réduit de manière importante l'apparition des crises de type absence chez les GAERS. Une étude pilote chez le GAERS a

confirmé un effet anti-épileptique robuste du LEV et révélé l'effet anti-épileptogène. Ceci nous a encouragé à explorer les propriétés neuromodulatrices du LEV chez le GAERS plus en détail. Notre travail a consisté à examiner l'effet du LEV sur les décharges pointe-ondes (spike and wave discharges, SWDs) chez le GAERS en fonction de l'âge. Le LEV a été administré de façon chronique avant l'âge d'apparition des SWDs dans l'EEG cortical. Notre travail montre que l'administration chronique du LEV supprime l'activité cérébrale épileptiforme chez les jeunes GAERS. De plus, l'effet anti-épileptique subsiste après la fin du traitement (PN61 – PN64), ce qui pourrait indiquer une inhibition des processus épileptogènes. Cet effet est cependant transitoire, car à l'âge de quatre mois (adulte) aussi bien les animaux non traités que les animaux traités présentent des SWDs sur l'EEG, qui ne diffèrent ni en durée ni en quantité. Des études plus poussées seront nécessaires pour définir une fenêtre temporelle optimale afin d'obtenir un effet plus permanent sur l'éptileptogenèse chez le GAERS.

La SNV est utilisée depuis 1988, et environ 30 000 patients épileptiques à travers le monde sont traités par cette méthode. L'expérience et la connaissance concernant la SNV augmentent rapidement, cependant plusieurs questions restent sans réponse. La SNV est utilisée pour les épilepsies généralisées et partielles, alors que les 'répondeurs' ne sont pas identifiés clairement. Un tiers des patients actuellement traités avec la SNV ne présente pas d'amélioration. Le mécanisme d'action de la SNV n'est pas encore parfaitement identifié. Une meilleure compréhension de ce mécanisme pourrait augmenter l'efficacité du traitement par l'identification des syndromes épileptiques spécifiques ou de formes d'épilepsie qui réagissent de manière positive à la SNV ou à l'optimalisation des paramètres de stimulation. Un grand nombre de structures intracérébrales sont potentiellement influencées par la stimulation des fibres vagales afférentes dans le cou. Les études animales initiales avec la SNV montraient des résultats prometteurs avec une diminution des anomalies critiques et intercritiques de l'EEG. Ces résultats ont constitué la base du développement futur de la SNV en tant que traitement de l'épilepsie chez l'homme. Néanmoins, l'efficacité de la SNV dans les études animales est évaluée en grande partie sur la base des modèles aigus (3-mercaptopropionate, pentylenetetrazol, électrochocs et application de pénicilline ou de strychnine). Les protocoles correspondants utilisent une stimulation appliquée immédiatement avant ou après l'induction des crises. Quelques études seulement ont évalué l'effet de la SNV sur des modèles animaux chroniques. Il est évident que des recherches animales supplémentaires, utilisant les modèles chroniques et des protocoles aussi bien aigus que chroniques, sont nécessaires. En outre, il nous manque des données fondamentales à propos de l'efficacité de la SNV sur l'épilepsie idiopathique. Des recherches sur l'efficacité de la SNV chez le GAERS, un modèle d'épilepsie absence idiopathique, peut aider à clarifier les principes généraux qui se trouvent à la base de la SNV. Dans ce modèle nous avons remarqué

un allongement temporaire de la durée de la crise suite à la SNV aigue. Cependant, lorsqu' une stimulation plus intense est utilisée, les SWDs typiques peuvent être interrompues immédiatement. Après une semaine de SNV chronique, les crises n'étaient pas différentes par rapport au groupe de contrôle. Nous émettons l'hypothèse qu'une longue période de SNV ou une stimulation avant les premiers symptômes est nécessaire pour influencer l'apparition d'un syndrome épileptique génétique. Deux heures d'administration quotidienne de SNV n'ont pas suffit à empêcher l'épileptogenèse chez les rats Fast, qui ont une sensibilité génétique à l'embrasement de l'amygdale. De même, des effets inhibiteurs aussi qu'excitateurs de la SNV sont observés dans ce modèle. Des profils de crises chez les rats ayant subi un « kindling » complet sont aggravés quand la SNV est donnée préalablement, tandis que la SNV immédiatement *après* le « kindling » arrive à empêcher les convulsions généralisées dans une sous-population du groupe SNV. De plus, la SNV ne paraît pas avoir d'effets secondaires cognitifs démontrable par le labyrinthe de Morris chez les rats Fast. La SNV entraîne une réduction du poids corporel des rats GAERS après deux semaines de traitement. Une hausse de poids typiquement associée au « kindling », est évitée chez les rats Fast (par absorption réduite de nourriture). Enfin, il est clair que la complexité de la SNV doit être étudiée en profondeur afin d'optimiser le traitement de patients épileptiques réfractaires.

Plusieurs études en imagerie fonctionnelle ont été conduites afin de rechercher les structures cérébrales activées ou inhibées par la SNV. Ces études montrent des changements cérébraux bilatéraux à la SNV unilatérale gauche, et soulignent le rôle important du thalamus et des structures temporales dans le mécanisme fonctionnel de la SNV. Il n'y a cependant aucun consensus quant à la nature des changements (excitation ou inhibition) ni quant à d'autres structures cérébrales qui pourraient être influencées par la SNV. Ces divergences peuvent être attribuée à plusieurs facteurs comme les différences entre plusieurs techniques d'imagerie (PET, SPECT, fMRI), les agents de contraste, les protocoles de scanning, les paramètres de stimulation, les médicaments, la durée de la maladie et la réaction au traitement. L'hétérogénéité des échantillons de patients est difficile à éviter. De plus, les données provenant d'individus en bonne santé sont impossibles à obtenir pour des raisons éthiques, la SNV étant une procédure assez invasive. C'est pour cela que nous avons examiné la possibilité d'explorer les effets de la SNV chronique et aigue sur le métabolisme glucosé du cerveau chez le rat en utilisant le micro-PET. Nous avons pu démontrer que la SNV chronique et aigue induisent des changements dans le métabolisme glucosé des régions cérébrales importantes pour le contrôle des crises comme l'hippocampe et le striatum. Notre étude pilote démontre que le « small animal PET » est une technique utile et prometteuse pour refléter l'activité du cerveau dans des études à long terme sur des rats.

Abbreviations

AD	afterdischarge
ADD	afterdischarge duration
ADT	afterdischarge threshold
AEDs	anti-epileptic drugs
CAP	compound action potential
DBS	deep brain stimulation
EEG	electro-encephalogram
EP	evoked potential
fMRI	functional magnetic resonance imaging
GABA	gamma-aminobutyric acid
GAERS	genetic absence epilepsy rats from Strasbourg
LEV	levetiracetam
MOA	mechanism of action
n.d.	not determined
NE	not effective
NS	not significant
NST	nucleus of the solitary tract
PET	positron emission tomography
PN	post natal day
PPS	perforant path stimulation
PTZ	pentylenetetrazole
RKRHS	rapid kindling with recurrent hippocampal seizures
SAS	sustained amygdala stimulation
SE	status epilepticus
SEM	standard error of the mean
SER	spontaneously epileptic rat
SD	standard deviation
SHS	sustained hippocampal stimulation
SNR	substantia nigra pars reticulata
SISWD	short irregular spike and wave discharge
SWD	spike and wave discharge
SPECT	single positron emission tomography
VNS	vagus nerve stimulation

Dankwoord

Dankwoord

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Curriculum vitae

Curriculum vitae

1. Personal details

Stefanie Dedeurwaerdere

Master in Science (biology)
Master in Laboratory Animal Science
PhD student

Nationality:	Belgian
Date and place of birth, age:	18/04/1979, Bruges (Belgium), 26 years
Marital status, number of children:	not married, no children
Private address and telephone:	Wijngaardstraat 36 9000 Ghent Belgium +32-476-603282
Work address:	Laboratory of Clinical and Experimental Neurophysiology Ghent University Hospital, Building B De Pintelaan 185, 9000 Ghent Belgium
Tel/Fax:	+32-9-2403355
E-mail:	Stefanie.Dedeurwaerdere@UGent.be
Position at work:	PhD student

2. University education

Doctoral research in Medical Science *Thesis: Neuromodulation in experimental animal models for epilepsy.* Faculty of Medicine and Health Sciences Promoter: Prof. Dr. P. Boon Co-Promoter: Prof. Dr. W. Verraes	Ghent University, Ghent, Belgium	01/10/2001-present
Master in Laboratory Animal Science FELASA Category D great distinction *Thesis: Anti-epileptogenic properties of levetiracetam in Genetic Absence Epilepsy Rats from Strasbourg* Faculty of Veterinary Medicine Promoter: Prof. Dr. P. Boon Co-Promoter: Prof. Dr. L. Van Ham	Ghent University, Ghent, Belgium	01/10/2002-01/07/2004
Basic Course in Laboratory Animal Science FELASA Category C	Ghent University, Ghent, Belgium	01/10/2002-06/02/2003
Master in Biology (option zoology) great distinction *Thesis: Vagus nerve stimulation in experimental animal models for epilepsy.* Faculty of Science, Department of Biology Promoter: Prof. Dr. P. Boon Co-Promoter: Prof. Dr. W. Verraes	Ghent University, Ghent, Belgium	07/07/2000-03/07/2001
Bachelor in Biology distinction	Ghent University, Ghent, Belgium	01/07/1998-29/09/1999

Practical training and projects performed at other research centers

The effect of vagus nerve stimulation in Fast kindling rats on memory, body weight, feeding behavior, epileptogenesis and seizures. Prof. D. McIntyre and Dr. K. Gilby	*Life Sciences Research Centre* Carleton University, Ottawa, Canada	12/07/2004-20/08/2004
Development of a new cuff-electrode for vagus nerve stimulation in rats and measurement of vagus nerve potentials induced by this spiral silicon-electrode in rats. Prof. C. Veraert and Dr. J. Delbeke	*GREN (Unité de Génie de la réhabilitation Neurale)* Université catholique de Louvain (UcLB), Brussels, Belgium	01/10/2003-01/10/2004
Training on stereotaxic implantation of depth electrodes and measuring of vagus nerve potentials evoked by a simple cuff-electrode in rats. Prof. W. Wadman	*Swammerdam Institute for Life Sciences* University of Amsterdam (UA), Amsterdam, Netherlands	14/10/2002-26/10/2002

3. Teaching experience

Practical course on reflexes, electromyography and nerve conduction Part of medical training	Ghent University, Ghent, Belgium	2002-2004
Supervise and guide master's students thesis work	Ghent University, Ghent, Belgium	
Tim De Smedt (Biology), Anticonvulsive and anti-epileptogenic properties of levetiracetam en valproic acid in rapid hippocampal kindling of the rat. *Awarded with the price Pierre Verkerck for best thesis*		2002-2003
Liesbeth Waterschoot (Biomedical Sciences), Stimulation of the nucleus reticularis of the thalamus in Genetic Absence Epilepsy Rats from Strasbourg (GAERS)		2003-2005
Liselotte Hardy (Biomedical Sciences), Chronic vagus nerve stimulation in Genetic Absence Epilepsy Rats from Strasbourg (GAERS)		2004-2006
Supervise and guide individual practical work of undergraduate biology students	Ghent University, Ghent, Belgium	March 2003
Correlation between Spike and Wave Discharges (SWD) and sleep/wake cycle in Genetic Absence Epilepsy Rats from Strasbourg (GAERS).		

4. Awards and grants

- **Predoctoral Research Grant** 011D9601 from the Ghent University Research Fund (B.O.F.): 01/10/2001-30/09/2005. *Neuromodulation in experimental animal models for epilepsy.*

- Travel Grant: European Neurological Society 2002, Berlin (Germany): 22/06/2002-26/06/2002. *Vagus nerve stimulation in Genetic Absence Epilepsy Rats from Strasbourg (GAERS)* (chairman epilepsy session 2 and oral presentation).

- **Young Investigators Travel Award:** American Epilepsy Society 2003, Boston (US): 05/12/2003-10/12/2003. *Anti-epileptic properties of levetiracetam in GAERS* (Platform presentation).

- **Travel Bursary:** 6th European Congress on Epileptology 2004, Vienna (Austria): 30/05/2004-03/06/2004. *Does levetiracetam has anti-epileptogenic properties in GAERS?* (oral presentation).

- **Boerhinger Ingelheim Fonds Travel Allowance:** research project at Carleton University 2004, Ottawa (Canada): 11/07/2004-20/07/2004. *Vagus nerve stimulation in Fast kindling rats.*

- Travel Grant: European Neurological Society 2005, Vienna (Austria): 18/06/2002-22/06/2002. *The effect of chronic levetiracetam administration on the development of spontaneous spike and wave discharges in Genetic Absence Epilepsy Rats from Strasbourg* (oral presentation).

5. Professional profile

- Junior researcher with extensive training and expertise in epilepsy research e.g. animal models of epilepsy (PTZ, GAERS, kindling models, status epilepticus model), anti-epileptic drugs, stereotaxic surgery, animal care, histology, electrical stimulation, electrophysiology, EEG, small animal PET, MRI and data analysis.
- Exceptional experience with vagus nerve stimulation in rats i.e. cuff-electrode fabrication, implantation, evaluation of efficacy in different animal models and mechanism of action.
- Dedicated scientist with outstanding organizational, troubleshooting and problem-solving skills.
- Computer-proficient in Microsoft Word, Microsoft Excel, Microsoft PowerPoint, Microsoft Outlook, SPSS, CorelDraw, Twin (Grass-Telefactor, Recording and Analysis Software, Hermes software package (imaging software: coregistration MRI and PET images) and some Linux.
- Knowledge of Dutch (native speaker), English (very good), French (good) and German (passive knowledge).

6. Conferences

Poster presentations (presenting author)

26th International Epilepsy Congress: *Electrophysiological and morphological evaluation of two cuff-electrodes for vagus nerve stimulation in rats.*	Paris, France	28/08/2005-01/09/2005
8th Workshop on the Neurobiology of Epilepsy – WONOEP VIII: *Vagus nerve stimulation decreases seizures in Fast rats, but does not have prophylactic effects on epileptogenesis.*	Villier-le-Mahieu, France	24/08/2005-26/08/2005
6th European Congress on Epileptology: *Does Levetiracetam have anti-epileptogenic properties in Genetic Absence Epilepsy Rats from Strasbourg?*	Vienna, Austria	30/05/2004-03/06/2004
Science Day (Department of Internal medicine): *Acute and Chronic* v*agus nerve stimulation in Genetic Absence Epilepsy Rats from Strasbourg.*	Ghent, Belgium	22/01/2004
VIIe Workshop On the Neurobiology Of Epilepsy (Wonoep): *Acute and chronic vagus nerve stimulation in Genetic Absence Epilepsy Rats from Strasbourg.*	Ericeira, Portugal	07/10/2003-11/10/2003
Science Day (Department of Internal medicine): *Vagus nerve stimulation in Genetic Absence Epilepsy Rats from Strasbourg.*	Ghent, Belgium	17/01/2003
Belgian Society for Fundamental and Clinical Physiology and Pharmacology: *Vagus nerve stimulation in Genetic Absence Epilepsy Rats from Strasbourg (GAERS).*	Ghent, Belgium	16/11/2002
From Brain Plasticity to Neuronavigation (*EKN*): *Vagus nerve stimulation in Genetic Absence Epilepsy Rats from Strasbourg: results of a pilot study.*	Ghent, Belgium	20/09/2002
55th Annual Meeting, American Epilepsy Society: *Vagus nerve stimulation in GAERS: results of a pilot study.*	Philadelphia, USA	29/11/2001-05/12/2001

Oral presentations

57th Annual Meeting, American Epilepsy Society: *Antiepileptogenic properties of Levetiracetam in Genetic Absence Epilepsy Rats from Strasbourg.*	Boston, USA	05/12/2003-10/12/2003
Conference on Neurology (EKN): *Acute and Chronic vagus nerve stimulation in Genetic Absence Epilepsy Rats from Strasbourg.*	Alden Biesen, Belgium	17/10/2003-18/10/2003
Belgian Society for Neuroscience: *Acute vagus nerve stimulation in Genetic Absence Epilepsy Rats from Strasbourg.*	Brussels, Belgium	19/05/2003-20/05/2003
Epilepsy and Sleep Update @Kempenhaeghe: *The effect of VNS and Levetiracetam on GAERS, a model of human absence epilepsy.*	Kempenhaeghe, Netherlands	28/03/2003
Belgian Society for Neuroscience: *Vagus nerve stimulation in Genetic Absence Epilepsy Rats from Strasbourg.*	Liege, Belgium	14/12/2002
European Neurological Society 2002: *Vagus nerve stimulation in Genetic Absence Epilepsy Rats from Strasbourg*.	Berlin, Germany	22/06/2002-26/06/2002
Multidisciplinar Workshop on Disease Modification: *Vagus nerve stimulation in Genetic Absence Epilepsy Rats from Strasbourg.*	Flinckheuvel, Belgium	26/04/2002

7. Reviewer for International Journals

- Seizure

- Acta Neurologica Belgica

8. Publications

Full articles in International Journals

1. Dedeurwaerdere,.S., Cornelissen,B., Van Laere,K., Vonck,K., Achten,E., Slegers,G. and Boon, P. (2005). Small animal positron emission tomography during vagus nerve stimulation in rats: a pilot study. Epilepsy Res In Press.
2. De Smedt,T., Vonck,K., Raedt,R., Dedeurwaerdere,S., Claeys,P., Legros,B., Wyckhuys,T., Wadman,W., Boon,P. (2005). Rapid kindling in preclinical anti-epileptic drug development: the effect of levetiracetam. Epilepsy Res In Press.
3. Dedeurwaerdere,S., Boon,P., De Smedt,T., Claeys,P., Raedt,R., Bosman,T., Van Hese,P., Van Maele,G., Vonck,K. (2005). Chronic levetiracetam treatment early in life decreases epileptiform events in GAERS, but does not prevent the expression of spike and wave discharges during adulthood. Seizure 14(6), 403-411.
4. Dedeurwaerdere,S., Vonck,K., Van Hese,P., Wadman,W., Boon,P. (2005). The acute and chronic effect of vagus nerve stimulation in "genetic absence epilepsy rats from Strasbourg" (GAERS). Epilepsia 46(Suppl 5), 94-97.
5. Vonck,K., Boon,P., Claeys,P., Dedeurwaerdere,S., Achten,E., Van Roost,D. (2005). Long-term deep brain stimulation for refractory temporal lobe epilepsy. Epilepsia 46(Suppl 5), 98-99.
6. Vonck,K., Dedeurwaerdere,S., De Groote,L., Thadani,V., Claeys,P., Gossiaux,F., Van Roost,D., Boon,P. (2005). Generator replacement in epilepsy patients treated with vagus nerve stimulation. Seizure 14, 89-99.
7. Vonck,K., Thadani,V., Gilbert,K., Dedeurwaerdere,S., De Groote,L., De Herdt,V., Goossens,L., Gossiaux,F., Achten,E., Thiery,E., Vingerhoets,G., Van Roost,D., Caemaert,J., De Reuck,J., Roberts,D., Williamson,P., Boon,P. (2004). Vagus nerve stimulation for refractory epilepsy: a transatlantic experience. J Clin Neurophysiol 21(4), 283-289.

8. Dedeurwaerdere,S., Vonck,K., Claeys,P., Van Hese,P., D'Havé,M., Grisar,T., Naritoku,D., Boon,P. (2004). Acute vagus nerve stimulation does not suppress spike and wave discharges in "genetic absence epilepsy rats from Strasbourg". Epilepsy Res 59(2-3), 191-198.
9. Vonck,K., Boon,P., Goossens,L., Dedeurwaerdere,S., Claeys,P., Gossiaux,F., Van Hese,P., De Smedt,T., Raedt,R., Achten,E., Deblaere,K., Thieleman,A., Vandemaele,P., Thiery,E., Vingerhoets,G., Miatton,M., Caemaert,J., Van Roost,D., Baert,E., Michielsen,G., Dewaele,F., Van Laere,K., Thadani,V., Robertson,D., Williamson,P. (2003). Neurostimulation for refractory epilepsy. Acta Neurol Belg 103(4), 213-217.
10. Lagae,L., Buyse,G., Ceulemans,B., Claeys,P., Dedeurwaerdere,S., de Meirleir,L., Hauman,R., Janssen,A., Schmedding,E., Verhelst,H. (2003). Vonck K. Anti-epileptogenesis research: the clinical relevance. Acta Neuro Belg 103(2), 78-82.
11. Van Hese,P., Martens,J.P., Boon,P., Dedeurwaerdere,S., Lemahieu,I., Van De Walle,R. (2003) Detection of SWD in the cortical EEG of GAERS. Phys Med Biol 48(12), 1685-1700.
12. Vonck,K., Van Laere,K., Dedeurwaerdere,S., Caemaert,J., De Reuck,J., Boon,P. (2001). The mechanism of action of vagus nerve stimulation for refractory epilepsy, the current status. J Clin Neurophysiol 18, 394-401.

Full articles in National Journals

13. Vonck,K., Dedeurwaerdere,S., Claeys,P., Goossens,L., Raedt,R., Vanhese,P., Van Roost,D., Caemaert,J., Achten,E., Vingerhoets,G., Thiery,E., De Reuck,J., Boon,P. (2003). Neurostimulatie voor refractaire epilepsie. De Agenda Psychiatrie 28, 11-12.
14. Dedeurwaerdere,S, Vonck,K., D'Havé,M., Grisar,T., Boon,P. (2002). Nervus vagus-stimulatie in "Genetic Absence Epilepsy Rats from Strasbourg", Deel 2: Resultaten en discussie. Neuron 8, 229-233.
15. Dedeurwaerdere,S., Vonck,K., D'Havé,M., Grisar,T., Boon,P. (2002). Nervus vagus-stimulatie in "Genetic Absence Epilepsy Rats from Strasbourg", Deel 1: Inleiding en methode. Neuron 6, 190-193.

Abstracts in International Journals

16. Dedeurwaerdere,S., Vonck,K., Van Den Broecke,C., Delbeke,J., Wadman,W., Boon,P. (2005) Electrophysiological and morphological evaluation of two cuff-electrodes for vagus nerve stimulation in rats. Epilepsia 46(Suppl 6), 205.
17. Legros,B., Hallez,H., Dedeurwaerdere,S., Raedt,R., De Smedt,T., Claeys,P., Pandolfo,M., Boon,P. (2005). Phototrombosis as a model for post-stroke epilepsy in rodents: results of chronic EEG recording. Epilepsia 46(Suppl 6), 198.
18. Wyckhuys,T.M.F., Raedt,R., Claeys,P., De Smedt,T., Pinxteren,J., Craeye,D., Smits,K., Vonck,K., Dedeurwaerdere,S., Waeytens,A., Van Den Broecke,C., Van De Kerckhove,B., Plum,J., Boon,P. (2005). Towards a cell therapy for temporal lobe epilepsy (TLE)? Epilepsia 46(Suppl 6), 355.
19. De Smedt,T., Raedt,R., Claeys,P., Dedeurwaerdere,S., Legros,B., Wyckhuys,T., Vonck,K., Boon,P. (2005). Antiepileptogenesis and antiepileptogenic properties of levetiracetam in rapid kindling. Epilepsia 46(Suppl 6), 369-370.
20. Dedeurwaerdere,S., Cornelissen,B., Vonck,K., Van Laere,K., Achten,E., Slegers,G., Boon,P. (2004). Micro-PET during vagus nerve stimulation in rats: a pilot study. Epilepsia 47(Suppl 7), 15.
21. Vonck,K., Dedeurwaerdere,S., Claeys,P., Achten,E., Van Roost,D., Boon,P. The mechanism of action of vagus nerve stimulation. Epilepsy Res 60(2-3), 150-152.
22. Dedeurwaerdere,S., Claeys,P., Raedt,R., De Smedt,T., Bosman,T., Van Hese,P., Van Maele,G., Vonck,K., Boon,P. (2004). Does levetiracetam have antiepileptogenic properties in genetic absence epilepsy rats from Strasbourg? Acta Neurol Belg 104(Suppl. 1), 58.

23. Legros,B., Hallez,H., Dedeurwaerdere,S., Raedt,R., De Smedt,T., Pandolfo,M., Boon,P. (2004) Development of a rodent model of post-stroke epilepsy: preliminary results. Eur J Neurol 11(Suppl. 2), 79.
24. Dedeurwaerdere,S., Claeys,P., De Smedt,T., Raedt,R., Vonck,K., Grisar,T., Boon,P. (2004) Levetiracetam has antiepileptogenic properties in "Genetic absence epilepsy rats from Strasbourg". Epilepsia 45 (Suppl. 3),112.
25. Dedeurwaerdere,S., Claeys,P., Raedt,R., De Smedt,T., Vonck,K., Grisar,T., Boon,P. (2004) Levetiracetam has antiepileptogenic properties in GAERS. Epilepsia 44(Suppl 9), 350.
26. Dedeurwaerdere,S., Raedt,R., Vonck,K., Claeys,P., Boon,P. (2003). Vagus nerve stimulation reduces body weight in Genetic Absence Epilepsy Rats from Strasbourg (GAERS). Epilepsia 44(Suppl 9), 327.
27. De Smedt,T., Raedt,R., Dedeurwaerdere,S., Vonck,K., Boon,P. (2003). The effect of levetiracetam and valproate in rapid kindling. Epilepsia 44(Suppl 9), 337.
28. Vonck,K., Boon,P., Claeys,P., Dedeurwaerdere,S., Achten,E., De Reuck,J., Caemaert,J. (2003). Amygdalohippocampal deep brain stimulation for refractory temporal lobe epilepsy. Epilepsia 44(Suppl 9), 329.
29. Vonck,K., Dedeurwaerdere,S., De Groote,L., Claeys,P., Baert,E., Caemaert,J., De Reuck,J., Boon,P. (2003). Indication and timing for pulse generator replacement in epilepsy patients treated with vagus nerve stimulation. J Neurol 250(Suppl.2), 17.
30. Dedeurwaerdere,S., Vonck,K., D'Havé,M., Naritoku,D., Grisar,T., Boon,P. (2003) Vagus nerve stimulation in genetic absence epilepsy rats from Strasbourg (GAERS). Fund Clin Pharmacol 17, 273.
31. Boon,P., Seys,L., Vonck,K., Dedeurwaerdere,S., D'Havé,M., Grisar,T., Claeys,P., De Reuck,J. (2003). The effect of levetiracetam in genetic absence epilepsy Strasbourg rats. Fund Clin Pharmacol 17, 271.
32. Dedeurwaerdere,S., Vonck,K., D'Havé,M., Naritoku,D., Grisar,T., Boon,P. (2003). Vagus nerve stimulation in GAERS. Acta Neurol Bel 1, 62.
33. Claeys,P., Vonck,K., Dedeurwaerdere,S., Boon,P. Reevaluation of intraperitoneal injection of pentylenetetrazol as a model for generalized epilepsy using digital video-EEG monitoring. Acta Neurol Bel 1, 62.
34. Dedeurwaerdere,S., Vonck,K., D'Havé,M., Naritoku,D., Grisar,T., Boon,P. (2002). Vagus nerve stimulation in genetic absence epilepsy rats from Strasbourg (GAERS). Acta Neurol Belg 4, 194.
35. Claeys,P., Vonck,K., Dedeurwaerdere,S., Boon,P. (2002). Reevaluation of intraperitoneal injection of pentylenetetrazol as a model for generalized epilepsy using digital video-EEG monitoring. Acta Neurol Belg 102, 192.
36. Dedeurwaerdere,S., Vonck,K., D'Havé,M., Naritoku,D., Grisar,T., Boon,P. (2002). Vagus nerve stimulation in genetic absence epilepsy rats from Strasbourg (GAERS). J Neurol 249(Suppl.1), 17.
37. Boon,P., Seys,L., Vonck,K., Dedeurwaerdere,S., D'Havé,M., Grisar,T., Claeys,P., De Reuck,J. (2002) The effect of levetiracetam in genetic absence epilepsy Strasbourg rats. Epilepsia 43(S8), 60.
38. Dedeurwaerdere,S., Vonck,K., D'Havé,M., Naritoku,D., Grisar,T., Boon,P. (2001). Vagus nerve stimulation in GAERS, results of a pilot study. Epilepsia 42(S7), 225-226.